The role of p53 in the embryonic stem cell response to DNA damage.

Sula Wendeline Corbet

PhD

The University of Edinburgh

1998

Accept where contributions from others have been specifically indicated, this thesis and the research described herein is solely my own work.

CONTENTS

Abstract.

The hypothesis that p53 deficiency enhances survival of DNA-damage bearing cells was investigated in embryonic stem (ES) cells with inactivation of one or both endogenous *p53* genes. ES cells were treated with a variety of DNA-damaging agents including UV light, γ-radiation and the topoisomerase I and II inhibitors, camptothecin and etoposide. Both topoisomerase inhibitors rapidly induced high levels of apoptosis in wild-type ES cells resulting in loss of most of the cell population within 48 hours. Consistent with results previously obtained from other cell types, the apoptotic response to etoposide was found to be p53-dependent at low doses but at the highest dose used p53-independent pathways became evident. Following UV-C irradiation, p53-protein was rapidly induced in wild-type cells and p53-dependent apoptosis followed within 8 hours, leading to death of the majority of cells within 36 hours. Treatment with ionising radiation led to enhanced expression of p53 but resulted in little induction of apoptosis irrespective of p53 status.

Evasion of apoptosis in the short term does not necessarily imply a continued capacity for growth and therefore long term survival, measured by clonogenic potential, was also examined. Following both UV and γ-irradiation, clonogenic survival of *p53*-null cells was significantly higher than survival of wild-type cells, particularly after high levels of DNA-damage where survival was enhanced more than ten-fold.

It has been predicted that a *p53*-null environment will result in an increased mutation rate due to loss of the normal apoptotic pathway and subsequent survival of cells bearing DNA damage. This prompted an investigation of the mutation frequency at the *hprt* locus after UV and γ-irradiation. After UV-irradiation, the *hprt* mutation frequency was found to be dependent upon UV dose and influenced by p53 status. In contrast, the incidence of *hprt* mutation following γ-irradiation did not differ significantly between wild-type and *p53*-null survivors.

In summary, the data confirm that p53 restricts the numbers of cells bearing mutations that survive DNA damage induced by either agent, albeit by mechanisms that differ. Thus, following ionising radiation there is a p53-dependent increase in the number of mutation bearing clones as a consequence of increased clonogenicity, whereas following treatment with UV-C changes to both mutation frequency amongst surviving cells and clonogenicity appear to be important.

Acknowledgements.

This project was supervised by Prof. A.H. Wyllie, Dr S. Gledhill and Dr A.R. Clarke whose enthusiasm, advice and support throughout were greatly appreciated.
Financial support for this research was provided by a grant from the Cancer Research Campaign.

I would like to thank Dr Bob Speed (MRC Human Genetics Unit, Edinburgh) and Dr Stephen Pye (Medical Physics, Western General Hospital, Edinburgh) for their tireless assistance with technical problems and data interpretation.

This study was undertaken at the Cancer Research Campaign Laboratories at the University of Edinburgh Department of Pathology and I would like to thank all those members of staff and students who assisted and encouraged me. In particular I would like to thank Robert Morris, Audrey Peters, Jill Bubb, Kate Cripps, Britta Dost, Lucy Curtis and Izabela Georgiades.

Finally I would like to thank my friends, family and especially my husband, Niall, for their patience and tolerance throughout this study.

List of abbreviations.

ADP	Adenosine monophosphate
ANOVA	Analysis of variance
APAF	Apoptotic protease activating factor
ATM	Ataxia-telengiectasia mutated
BCL2	B-cell lymphoma gene 2
BH	BCL2 homology domain
bp	Base-pair
53BP2	p53 binding protein 2
CARD	Caspase recruitment domain
CD95	Cell surface determinant 95
CDK	Cyclin dependent kinase
cDNA	Complimentary DNA
ced	Cell death abnormal
CS	Cockaynes syndrome
dCTP	Deoxycytosine triphosphate
ddNTP	Dideoxynucleoside triphosphate
DDW	Doubly distilled water
DED	Death effector domain
Dlb	Dolichos biflorus
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNAPK	DNA dependent protein kinase
dNTP	Deoxynucleoside triphosphate
dsb	Double strand break
DTT	Dithiothreitol
EBV	Epstein Barr virus
EC	Embryonic carcinoma
EDTA	Ethylenediaminetetraacetic acid
ES	Embryonic stem

FADD	Fas-associated protein with death domain
FCS	Foetal calf serum
FISH	Fluorescent *in situ* hybridisation
FITC	Fluorescein isothiocyanate
GMP	Guanine monophophate
HAP1	Human AP endonuclease 1
HAT	Hypoxanthine, aminopterin and thymidine containing cell culture medium
HPV	Human papilloma virus
HPRT	Hypoxanthine phosphoribosyl transferase
ICE	Interleukin-1β-converting enzyme
IGFBP3	Insulin-like growth factor binding protein 3
IMP	Inosine monophosphate
kb	Kilobase-pairs
lacI	Galactosidase inhibitor
LIF	Leukaemic inhibitory factor
MSH2	MutS homologue 2
NBS	Newborn calf serum
neo	Neomycin resistance gene
NER	Nucleotide excision repair
NFκB	Nuclear factor κB
NMR	Nuclear magnetic resonance
NSS	Normal swine serum
4NQO	4-nitroquinoline 1-oxide
PALA	*n*-phosphonacetyl-L-aspartate
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PI	Phosphotidyl inositol
PT	Permeability transition

RAIDD	Receptor-associated independent death domain
REF1	Redox factor 1
RNA	Ribonucleic acid
RPA	Replication protein A
SDS	Sodium dodecyl sulphate
ssb	Single strand break
SSC	Saline, sodium citrate buffer
TAF	TBP-associated factor
TBP	TATA box binding protein
TBS	Tris buffered saline
TBST	Tris buffered saline with Tween
TE	Tris, EDTA buffer
TFIID	Transcription factor IID
6-TG	6-amino-6-mercaptopurine
TNE	Sodium, Tris and EDTA buffer
TNF	Tumour necrosis factor
TVP	Phosphate buffered trypsin/versine
UVC	Ultra violet irradiation with a wavelength of 254 nm
WT1	Wilms tumour 1
XP	Xeroderma pigmentosum

CHAPTER ONE

INTRODUCTION

1.1 A pathway to death.

The process of apoptosis was first described as a cellular process characterised by defined structural changes. Developments in the past five years have led to the description of a biochemical pathway dependent on the activity of a set of genes which are also familiar in cancer. One major feature of this pathway is the existence of a terminal component that is common to a wide variety of stimuli, both pathological and physiological. The question therefore arises of what happens when genes involved in the apoptosis pathway are deficient? Do cells die by another route, or do they survive, and if so are they normal or perhaps the precursors of cancer cells? This thesis attempts to answer some of these questions. It studies apoptosis in response to DNA (deoxyribonucleic acid) injury, in particular to two agents with differing modes of action, and analyses the effect on apoptosis of a single genetic change: the removal of p53. In addition, it attempts to identify conditions in which the absence of p53 leads to increased survival, both in terms of escape from immediate apoptosis and continued proliferation thereafter. Finally, it examines the phenotype, with regard to the acquisition of mutations, of those cells which without the loss of p53 would otherwise have been deleted from the population.

As a background to these studies, it is necessary to describe the cell biology of apoptosis - both the common terminal pathway and some of the regulatory genes, in particular p53, concerned with coupling apoptosis to DNA damage.

1.2 Apoptosis.

It has long been recognised that cell death is an essential process in embryonic development (Glucksmann, 1951; Saunders and Fallon, 1966) but the discovery that this process, with its typical structural changes, also occurred in cells dying from a

wide variety of causes led to the definition of the term apoptosis (Kerr *et al.*, 1972). The importance of the apoptotic process to the survival of both individual cells and the organism as a whole has only been recognised relatively recently. It is now known that it plays a role in a broad range of physiological processes, the failure of which often results in disease.

1.2.1 The physiological role of apoptosis.

The earliest reports of cell death, now over a century old (Beard, 1896, in Milligan and Schwartz, 1997), were from observations of embryonic development. It is now clear that apoptosis plays an essential role in many, if not all, developmental processes and its failure results in a wide variety of congenital abnormalities (reviewed by Milligan and Schwartz, 1997). During the development of the foot, the interdigital cells are removed by apoptosis and failure of this process results in excess skin, or webbing, between the digits (Garcia-Martinez *et al.*, 1993). Similarly, apoptotic regression of the midline epithelium is required for formation of the palate (Pratt and Greene, 1976; Mori *et al.*, 1994). Even in adult life, apoptosis continues to remove cells that are surplus to requirements. Examples include; the involution of the mammary gland after lactation, regression of the adrenal cortex in response to decreases in adrenocorticotrophic hormone and breakdown of the uterine endometrium during the menstrual cycle (Wyllie *et al.*, 1973; Hopwood and Levison, 1976; Walker *et al.*, 1989; Strange *et al.*, 1992).

Another crucial physiological function for apoptosis is in the immune system. During development of the immune system, the vast majority of T and B lymphocytes die by apoptosis. Here apoptosis occurs by default in the absence of survival signals. In the case of B cells, survival signals are only produced by the expression on the cell surface of an antigen receptor and successful signal transduction, thus eliminating cells without functional antigen receptors (Tonegawa, 1983). A similar process occurs in T cells, with cells failing to make successful T cell receptor gene rearrangements again being deleted by apoptosis (Raulet *et al.*, 1985). Negative selection of autoreactive T and B cells also occurs by apoptosis

(Miller and Heath, 1993; Nemazee and Burki, 1989), as does the removal of activated mature T and B cells once their purpose has been served (Russell, 1995). Cytotoxic T lymphocytes and natural killer cells are specialised immune cells which have the ability to destroy target cells. Target cells are recognised by interactions between receptors on the surface of the killer cell and antigens on the surface of the target. The killer cell is able to induce apoptosis in the target cell, either via cell-surface determinant 95 (CD95) signalling or exocytosis of granules containing perforin and granzymes (Rouvier *et al.*, 1993; Darmon *et al.*, 1995). Inability to produce functional antigen receptors, for example due to loss of DNA-dependent protein kinase activity (DNA-PK, Blunt *et al.*, 1995), results in severe combined immunodeficiency. In this case, disease is associated not with failure of the death pathway but is a normal response to non-functional receptor gene rearrangements. Immunological disorders can also result from abnormalities in apoptosis in lymphoid cells, for example mutations in *CD95* cause an autoimmune lymphoproliferative disorder. Thus, the immune system is dependent upon a finely balanced equilibrium between proliferation and apoptosis and any perturbations to the system have serious repercussions for its effectiveness.

1.2.2 Apoptosis and cell injury.

Apoptosis is also a critical component of the cellular response to injury. Apoptosis may be initiated in response to a broad range of types of cellular injury including DNA damage, mitochondrial injury, cell membrane injury and viral infection. The question arises of how cell injury initiates apoptosis and to what extent does the cellular response to injury share a common pathway with the physiological induction of apoptosis.

1.2.3 Structural changes during cell death.

The first evidence for a single common pathway to apoptosis was morphological. During apoptosis cells undergo a well characterised series of physical changes. With the onset of apoptosis, cells lose contact with neighbouring cells and a period of intense membrane-blebbing ensues. This is followed by condensation of both the

cytoplasm and nuclear chromatin. The nuclear chromatin becomes peripheralised, forming dense cap-like structures along the inner margin of the nuclear envelope. This is accompanied by redistribution of the nuclear pores and breakdown of the nuclear lamina. Finally, the cell forms a cluster of membrane bound apoptotic bodies which, within tissues and sometimes also in vitro, are subsequently phagocytosed by either neighbouring cells or specialist phagocytes. Apoptosis in many, but not all, cells is also characterised by fragmentation of genomic DNA by an endogenous endonuclease. Genomic DNA is cleaved first into large fragments of between 50 and 300 kilo base-pairs (kb), described as domain cleavage, and subsequently within the internucleosomal linker regions resulting in a DNA ladder of fragments differing in size by around 180 base-pairs (bp). One of the most striking features of apoptosis is the speed with which cells are removed from tissues: the entire process is normally completed within a few hours (Kerr, 1971; Wyllie, 1980; Allen, 1987; Kerr *et al.*, 1987; Lazebnik *et al.*, 1993).

1.2.4 The genetic regulation of apoptosis.

In the previous section the structural changes which occur during the apoptotic process have been discussed but how is this process initiated and what are the effector molecules? Our understanding of the regulation of apoptosis in mammals has been greatly aided by a series of painstaking experiments elucidating the genetic control of apoptosis during the development of the nematode worm, *Caenorhabditis elegans* (reviewed by Miura and Yuan 1996). During nematode development, 131 cells die by apoptosis resulting in a worm incorporating only 1090 cells, each of which can be examined by light microscopy. Each cell destined to die does so at a time precisely predetermined by a genetically defined programme. Disruption of this genetic pathway by mutagenesis has resulted in the identification of 14 genes involved in the death pathway. These include genes involved in the commitment of the cell to the death pathway, execution of cell death, engulfment of the dead cell and its degradation. Probably the most significant of these are the three *Caenorhabditis elegans* death (*ced*) genes *ced-3* , *ced-4* and *ced-9*. Both *ced-3* and *ced-4* are necessary and sufficient to initiate apoptosis (Xue *et al.*, 1996; Hugunin *et al.*, 1996;

Yuan and Horvitz, 1992) whilst *ced-9* inhibits the death pathway and is necessary for cell survival (Hengartner *et al.*, 1992). Attempts to find the mammalian homologues of these genes led to the discovery of homology between *ced-3* and interleukin-1β-converting enzyme (*ICE* or *caspase-1*), and *ced-9* and a gene involved in human B-cell lymphomas (*BCL2*).

1.2.4.1 Caspases, the effector molecules of the apoptotic pathway.

The first member of the caspase family to be identified was ICE (now called caspase 1), an enzyme responsible for the processing of the pro-inflammatory cytokine, pro interleukin-1β (Thornberry *et al.*, 1992). The discovery that this gene was homologous to *ced-3* (Yuan *et al.*, 1993), prompted an intensive search for other mammalian homologues and resulted in the discovery of a whole family of related genes. To date, ten human caspases have been identified (reviewed by Thornberry, 1997a).

Caspases exist as proenzymes requiring proteolytic cleavage to an active form. The proenzyme is composed of two subunits, an N-terminal prodomain of variable length and in some enzymes, a linker region between the two subunits. Proteolytic cleavage of the enzyme is thought to occur at Aspartic acid residues and results in removal of the prodomain and separation of the two subunits, with loss of the linker region where present. There is evidence to suggest that the mature form of the enzyme is a tetramer formed from two of the smaller subunits and two large subunits (Thornberry *et al.*, 1992; Wilson *et al.*, 1994; Walker *et al.*, 1994). Phylogenetic comparisons of the gene family have suggested that the enzymes may be grouped into two, or possibly three, subfamilies (reviewed by Thornberry, 1997a). All caspases have a requirement for an aspartic acid residue at the P1 position; the first aa N-terminal to the cleavage site. Differences between family members include variations in the size of the prodomain and subunits, and differences in substrate specificity. Differences in substrate specificity and the prodomains probably account for the functional differences between family members. Several caspase family members have been found to encode a number of splice variants. Isoforms of some family members,

which include full-length and truncated proteins, have opposing functions as inducers and inhibitors of apoptosis, possibly via a dominant negative inhibition mechanism.

There are several lines of evidence indicating that at least some caspases are mediators of mammalian apoptosis. First, cleavage and consequent activation of certain caspases correlates temporally with the appearance of the apoptotic phenotype (Schlegel *et al.*, 1996; Duan *et al.*, 1996; Orth *et al.*, 1996). Second, caspases appear to be responsible for the proteolytic cleavage of a number of proteins known to be cleaved during apoptosis and involved in cellular homeostasis, repair and the maintenance of cell structure (reviewed by Thornberry, 1997a). Third, studies with viral and synthetic caspase inhibitors indicate that these inhibitors can prevent cell death in response to a variety of inducing agents, in a wide range of cell types (for examples, see Gagliardini *et al.*, 1994; Lazebnik *et al.*, 1994; Nicholson *et al.*, 1995; Enari *et al.*, 1995). Finally, studies of gene knockout mice indicate that caspase 3 (CPP32/yama/apopain) is essential for apoptosis during the development of the brain and nervous system (Kuida *et al.*, 1996).

Although it is clear that caspases do play a role in apoptosis, there is also considerable evidence to suggest a high degree of redundancy among family members. The strongest evidence comes from knockout mice. Although, as previously mentioned, *caspase 3* knockout mice show severe defects in apoptosis of neural tissues, *caspase 1* null mice show no defects in apoptosis and mice deficient in caspase 2 show only limited resistance to apoptosis in B-cells and oocytes (Kuida *et al.*, 1995; Kuida *et al.*, 1996; Bergeron *et al.*, cited in Dost, 1998). Duplication among some family members of substrate specificities and the co-expression of multiple caspases in many tissues is also consistent with some redundancy.

Current evidence indicates that caspases play roles in both the regulation and execution of apoptosis. In CD95 mediated apoptosis, caspase 8 has been implicated in upstream signalling events whilst caspase 3 is involved in the effector phase of cell death. Caspase 8 physically interacts with the CD95 receptor complex linker protein

via dimerisation between the death domain located within its own prodomain and the death domain of the linker (Boldin *et al.*, 1996; Muzio *et al.*, 1996). Although the mechanism is not yet clear, it is thought that this interaction results in cleavage and activation of caspase 8, which then cleaves and activates other caspases, resulting in a caspase cascade. Death domains have also been found in the prodomains of caspases 2 and 10, suggesting a similar function for these enzymes (Fernandes-Alnermi *et al.*, 1996). In support of the theory of a cascade of protease activity, various members of the caspase family have been shown to have protease activity against other family members and when overexpressed in bacteria are able to catalyse their own activation. In addition, the caspase inhibitor CrmA has been shown to prevent the activation of caspases 3 and 7 although CrmA does not directly interact with caspase 7 (Zhou *et al.*, 1997; Chinnaiyan *et al.*, 1996). It has recently been shown that the prodomain of caspase 1 is required for its dimerisation and autoactivation (Van Criekinge *et al.*, 1996) and it has been speculated that those caspases with long prodomains may play a regulatory role in apoptosis in which they activate those caspases with short prodomains that may be unable to autoactivate in cells (Thornberry, 1997b). The question still remains as to how this cascade of protease activity is initiated. In the less complex pathway of *C. elegans*, it has been suggested that CED-4 fulfils this role.

1.2.4.2 *ced 4* and its mammalian homologue, *Apaf1*.

The *C.elegans* gene *ced-4* is required for *ced-3* function and apoptosis. *ced-3* is a cysteine protease with aspartic acid specificity (caspase, see section 1.2.4.3) that exists as a proenzyme requiring activation by cleavage. It has been proposed that this activation is the function of CED-4. Recent studies have shown that CED-3 and CED-4 are able to physically interact (Chinnaiyan *et al.*, 1997b; Irmler *et al.*, 1997), probably via their N-terminal, caspase recruitment domains (CARD) (Hofmann *et al.*, 1997). CED-9 is also able to bind to CED-4 and prevents its activation of CED-3 (Chinnaiyan *et al.*, 1997b; James *et al.*, 1997; Spector *et al.*, 1997; Wu *et al.*, 1997). Several mammalian caspase activating proteins involved in the transduction of apoptotic signals from the TNF (tumour necrosis factor) receptor family have been

identified. These include FADD (fas-associated protein with death domain) which binds certain caspases via its death effector domain (DED) and RAIDD (receptor associated independent death domain) which binds through a CARD domain (Hofmann *et al.*, 1997). However, these proteins share little homology with CED-4 and are poorly blocked by BCL2. The first true homologue of CED-4, APAF1 (apoptotic protease activating factor 1), was reported only very recently (Zou *et al.*, 1997). It has an N-terminal CARD domain and a 320 aa central region sharing 22% identity and 48% similarity with CED-4. APAF1 is able to bind cytochrome c and in its presence and the presence of ATP and APAF3 (an unknown 45 kDa protein) activates the CED-3 homologue, caspase 3. Very little is yet known about APAF1 and its role in mammalian apoptosis but it is clear that at least part of that role is the activation of caspases. As previously discussed, the product of the *C. elegans* gene *ced-9* is thought to bind to CED-4 and prevents its activation of CED-3. One possible mechanism by which BCL2 might inhibit the APAF1 mediated activation of caspase 3 would be by preventing cytochrome c release from the mitochondria. In support of this theory, release of cytochrome c in response to apoptotic stimuli is blocked in cells overexpressing BCL2 (Kluck *et al.*, 1997; Yang *et al.*, 1997).

1.2.4.3 The *BCL2* gene family.

BCL2 was first identified by its involvement in B-cell lymphomas where its expression is commonly altered by the t(14;18) translocation (Tsujimoto *et al.*, 1985). This translocation places the *BCL2* gene in close proximity to enhancer elements of the immunoglobulin heavy chain locus, resulting in increased expression of the BCL2 protein and enhanced cellular survival (Vaux *et al.*, 1988). *BCL2* is only one of a large family of related genes sharing up to four (BH1 to BH4) highly conserved regions of homology (reviewed by Hale *et al.*, 1996; Kroemer *et al.*, 1997; Kroemer 1997 and Brown 1997). Functionally, the family can be divided into two groups; death inducing and death inhibiting proteins. Death inhibiting proteins include products of the genes *BCL2, Bclxl, Bcl-w, Bragl, Bfll, Mcll* and *A1* and death inducing proteins include the products of *Bclxs, Bax, Bak, Bad, Bid, Bik* and *Hrk*. Family members differ structurally and also in their expression patterns.

Gene knock-out studies have helped to clarify the role of some *BCL2* family members in both embryonic development and the adult mouse. *Bcl2*-null mice survive to birth but die shortly after showing polycystic kidney disease and massive cell death in the thymus and spleen (Veis *et al.*, 1993; Nakayama *et al.*, 1994; Kamada *et al.*, 1995). *Bclx*-null mice die during embryonic development (day 13), apparently due to extensive apoptotic death in the nervous and haematopoeitic systems (Motoyama *et al.*, 1995). The least affected phenotype is that of *Bax* knock-out mice which survive to adulthood but show male sterility and thymocyte and B-cell hyperplasia (Knudson *et al.*, 1995). These results suggest substantial functional redundancy among *BCL2* family members but indicate that particular members are essential to some cell types at specific times.

Many of the *BCL2* family proteins bear a carboxyterminal transmembrane region and are predominantly localised within the outer mitochondrial membrane (Monaghan *et al.*, 1992; Jacobson *et al.*, 1993; Krajewski *et al.*, 1993; Muchmore *et al.*, 1996). BCL2 shows a discontinuous distribution in the mitochondrion consistent with an association with the mitochondrial megachannel or permeability transition (PT) pore (Zoratti and Szabo, 1995; Kroemer *et al.*, 1997). Deletion of the transmembrane domain in BCL2 abrogates or diminishes its ability to inhibit apoptosis (Tanaka *et al.*, 1993; Hockenberry *et al.*, 1993). This loss of function can be restored by exchanging the transmembrane domain for an equivalent sequence from either yeast Mas-p70 or cytochrome b5 (Zhu *et al.*, 1996). Similar experiments in yeast have shown that membrane targeting is also crucial for the apoptosis-promoting activity of BAX (Zha *et al.*, 1996a). Thus, some BCL2 related proteins appear to require mitochondrial localisation to regulate apoptosis.

The *BCL2* gene family is characterised by the ability to form homodimers and heterodimers with other family members, although heterodimerisation is often selective. Over-expression of *BCL2* protects cells from apoptosis induced by a variety of, but not all, inducing agents in many cell types. In addition, it can

substitute for CED-9 in suppressing cell death in *C. elegans*. Over-expression of the death-inducer *BAX* on the other hand, induces apoptosis and can antagonise the protective function of BCL2. BAX was isolated as a BCL2 binding partner in immunoprecipitation studies (Oltvai *et al.*, 1993) suggesting that the formation of BCL2/BAX heterodimers may play a role in the control of apoptosis. In support of this hypothesis, BCL2 mutations in key regions of BH1 and BH2 that compromise binding with BAX result in loss of the ability of BCL2 to inhibit apoptosis (Yin *et al.*, 1994). However, although this theory is consistent with the data, it is possible that the true mechanism is more complex as most studies to date have only examined interactions between one or two BCL2 family members in any one experimental system. It is now clear that other family members also play a role in the regulation of apoptosis and it is not possible to determine the effects of loss of BCL2/BAX binding on these proteins and other interacting proteins not related to the BCL2 family. Indeed, several mutations that disrupt the binding of BCLX-L to BAX have been reported that only partially abrogate its death repressor activity (Cheng *et al.*, 1996). Experiments suggest that the BH3 domain of death agonists is required both for binding to BCL2 and BCLX-L and to promote apoptosis (Zha *et al.*, 1996b; Hunter and Parslow, 1996). However, a number of experiments on BH3 mutants of the death agonist BID indicate that these two functions are controlled separately (Wang *et al.*, 1996a). BID, like BIK but unlike BAX, contains only the BH3 domain and not BH1 and BH2 and may represent a death domain ligand for the membrane-bound receptor BAX (Boyd *et al.*, 1995; Wang *et al.*, 1996a). The BH4 domain is conserved among the death antagonist members of the BCL2 family but is either lacking or ill-conserved in all but one (BCLX-S) death agonists. Deletion of this domain does not affect dimerisation but results in either loss of function or conversion of death antagonists to death agonists (Hunter *et al.*, 1996). Intriguingly, BH4 is required for the binding of a number of death regulatory proteins not related to the *BCL2* gene family. These include BAD, RAF1, CED-4, and calcineurin (Wang *et al.*, 1996b; Chinnaiyan *et al.*, 1997a; Shibasaki *et al.*, 1997).

A further level of complexity is added to the system when we consider the effects of post-translational modification on activity of BCL2 family members. Serine phosphorylation of BCL2 family members is known to affect their activity. Deletion of a proposed negative regulatory loop, between BH4 and BH3, in BCL2 enhances its ability to inhibit apoptosis (Uhlmann *et al.*, 1996). In its unphosphorylated form, BAD heterodimerises with both BCL2 and BCLX-L, thereby promoting apoptosis. In the presence of the survival factor IL-3, BAD is phosphorylated at serine residues and bound to the 14-3-3 protein, thus rendering it unavailable for binding to BCL2 or BCLX-L. Withdrawal of IL-3 results in dephosphorylation of BAD and apoptosis. Thus, substitution of phosphorylation sites in BAD favours its death inducing activity (Zha *et al.*, 1996c). BCL2 can also phosphorylate BAD via the targeting of its binding partner, RAF1, to the mitochondrial membrane (Wang *et al.*, 1996b).

BAX overexpression has been associated with a wide variety of apoptosis-related changes and a correspondingly large number of theories have been suggested for its mechanism of action. Only recently has it become clear that one of the first and most universal manifestations of the apoptotic process is disruption of mitochondrial membrane function. These changes include dissipation of the mitochondrial transmembrane potential and/or the release of mitochondrial proteases and cytochrome c (Kroemer *et al.*, 1995; Zamzami *et al.*, 1995; Liu *et al.*, 1996; Susin *et al.*, 1996; Yang *et al.*, 1997; Kroemer *et al.*, 1997). It is thought that the collapse of the mitochondrial transmembrane potential may be due to the opening of the mitochondrial PT pores, the consequences of which could account for all of the metabolic changes associated with apoptosis (Kroemer *et al.*, 1997; Kroemer, 1997). This theory is supported by studies showing that inhibition of PT abrogates all mitochondrial and post-mitochondrial modifications associated with apoptosis in some model systems but it is still not clear whether PT is influenced directly or indirectly by *BCL2* family members (Zamzami *et al.*, 1995; Kroemer *et al.*, 1997). Intriguingly, recent NMR (nuclear magnetic resonance) and X-ray studies on the structure of BCLX-L have revealed that there are seven alpha helices, the alignment of which is similar to the membrane insertion domain of a group of bacterial toxins

and suggests that this protein might act as some kind of pore or membrane channel (Munchmore *et al.*, 1996).

BCL2 is not able to inhibit apoptosis induced by all stimuli. It can inhibit p53-dependent death in response to DNA-damage and death of growth factor deprived cells but does not affect apoptosis induced by the T-cell protease, granzyme B (Chiou *et al.*, 1994; Vaux *et al.*, 1992). These and other data allow us to place the BCL2 gene on a point of the apoptosis pathway upstream of the effector proteases but downstream of many of the signalling molecules.

In summary, it appears that members of the BCL2 family interact with each other to form a complex network of homo and heterodimers resulting in a dynamic equilibrium of life and death signals. The details of this network are still largely unknown but it seems that localisation to the mitochondrial membrane of some members of the BCL2 family is essential for their efficient action and that post-translational modification of proteins may also play a role. The mechanism by which BCL2 family proteins effect their regulation of apoptosis is also unclear but may, either directly or indirectly, involve loss of mitochondrial transmembrane potential. Loss of transmembrane potential might be expected to cause the release of cytochrome c from the mitochondrion, which could, in the presence of the CED-4 homologue, APAF1, and its cofactors (APAF3 and ATP), result in the activation of caspase 3 or other members of the caspase family, thus initiating the caspase cascade and resulting in the induction of the structural changes associated with apoptosis.

1.2.4.4 Transcriptional signals for the activation of apoptosis.

The gene *reaper* is required for efficient apoptosis in response to both cell injury and physiological stimuli in *Drosophila* and in its absence sensitivity to these stimuli is reduced by around a thousand fold (White *et al.*, 1996). Reaper shows some structural homology to the CD95 death domain and may link into the same terminal effector cascade as CD95 (Golstein *et al.*, 1995). However, no mammalian homologue for *reaper* has been reported yet. The proto-oncogene *c-myc* is an

immediate-early growth response gene required for cell cycle progression. However, overexpression of c-myc in serum starved fibroblasts results in the initiation of apoptosis (Evan *et al.*, 1992). Both the mitogenic and apoptotic functions of *c-myc* seem to require the same gene domains. In addition, there is evidence that serum starved fibroblasts enter S-phase prior to apoptosis (Hermeking and Eick, 1994). These findings have led to the hypothesis that *c-myc* induces a state of readiness in these fibroblasts which may result in either cell division or cell death depending on the availability of local growth factors (Harrington *et al.*, 1994). The oncosuppressor gene *Rb1* is also involved in the control of cell cycle progression and is essential for exit from the cycle into G_0. When subjected to DNA damage, fibroblasts would normally leave the cell cycle and enter G_0 arrest but in the presence of either adenovirus E1A protein or human papillomavirus (HPV) E7 protein, both of which inactivate RB1 and thus prevent cell cycle exit, these cells undergo apoptosis (Debbas and White, 1993; Slebos *et al.*, 1994). In its active, hyperphosphorylated form, RB1 binds to and inhibits the activity of the E2F family of transcription factors which are believed to mediate entry into S phase. Overexpression of E2F-1 also results in the induction of apoptosis in serum starved fibroblasts (Shan and Lee, 1994; Qin *et al.*, 1994). *p53* and *IRF1* are both tumour suppressor genes that can signal apoptosis under certain circumstances. The effects of these two genes appear to be interdependent and both share the ability to initiate either cell cycle arrest or apoptosis (see section 1.3.5; Canman *et al.*, 1995; Tamura *et al.*, 1995). What factors determine which of these end-points is chosen is still unclear.

1.3 The cellular response to DNA damage.

A great variety of DNA lesions are of importance to the cell as they may result in mutations or have the potential to disrupt transcription or replication. It is therefore not surprising that there are sophisticated molecular systems for recognition of such lesions and for coupling them to repair pathways. Nucleotide excision repair (NER) is one of the most important cellular repair pathways and is involved in the repair of a wide variety of bulky DNA lesions including those induced by UVC-irradiation and a broad range of chemicals. The primary lesions induced by UVC-irradiation include

6-4 photoproducts and cyclobutane pyrimidine dimers (Setlow and Carrier, 1966; Lippke *et al.*, 1981). These lesions result in distortions of the DNA helix which are recognised by the proteins XPA (xeroderma pigmentosum complementation group A) and, in the case of 6-4 photoproducts, XPE (xeroderma pigmentosum complementation group E, Chu and Chang, 1988; Chu and Chang, 1990; Robins *et al.*, 1991). The mismatch repair pathway is involved in the repair of mis-paired bases formed during DNA replication, recombination and as a result of DNA damage. Here lesions are recognised primarily by the MSH2 (MutS homologue 2) protein which is also involved in recruitment of the repair complex (reviewed by Kolodner, 1996). p53 has also been shown to bind to insertion/deletion mismatches consisting of one or several extra bases on one strand but has little affinity for the classical type of mismatch recognised by MSH2. Depurination of DNA is probably the most common type of spontaneous DNA lesion and also results from oxidative damage generated by ionising radiation. These lesions are usually repaired by pathways involving AP endonucleases like the human AP endonuclease 1 (HAP1) and redox factor 1 (REF1) (reviewed by Boulikas, 1996). A number of nuclear proteins are able to recognise DNA single and double-strand breaks. These include poly(ADP-ribose) polymerase (PARP), the c-terminal domain of p53, DNA-PK and DNA ligases. PARP is rapidly induced by both single and double strand breaks and, until recently, was thought to be involved in DNA repair. However, the generation of PARP knockout mice has indicated that this protein is not required for either NER or base excision repair (Wang *et al.*, 1995). Of particular relevance to this study, is the repair of DNA double-strand breaks which are thought to be the most biologically significant of the lesions resulting from ionising radiation. Recognition and repair of this type of lesion involves DNA-PK (reviewed by Jackson, 1996). DNA-PK is a multi-protein complex consisting of a catalytic subunit (DNA-PK_{CS} or XRCC7) and a DNA binding component, KU (also called XRCC5). DNA-PK_{CS} shares homology with a subgroup of the phosphatidylinositol (PI) 3-kinase family. Another subgroup member is the recently cloned ATM gene which is mutated in individuals suffering from ataxia-telangiectasia (AT) (reviewed by Jackson, 1996). AT cells display elevated levels of genomic instability and are hypersensitive to ionising radiation and

other agents that induce double-strand breaks, suggesting the involvement of ATM in DNA-damage recognition or repair. AT cells also show a delayed p53 response to irradiation and have impaired function for both the G_1/S and G_2/M checkpoints. Whether or not ATM plays a direct role in DNA repair is still unclear but there is some evidence to suggest that ATM is in involved in the regulation of nuclear factor κB (NFκB) activity. In addition, it is tempting to speculate on a linkage between ATM and p53 and it has been suggested that p53 might act as a DNA damage targeting subunit for ATM (Jackson, 1996).

Many of the molecules involved in DNA-damage recognition and repair also link into apoptotic pathways. The precise nature of this link is not fully understood but this thesis is concerned with one pathway in particular - the p53-dependent DNA damage response. A wide variety of DNA-damaging agents have been shown to induce apoptosis in numerous cell types. In many experimental systems, the tumour suppressor gene *p53* has been shown to play a central role in this signalling pathway. However, this is not the only function of p53. It also has a role in cell cycle regulation and has been implicated in differentiation, DNA damage recognition and DNA repair.

1.3.1 p53 protein structure and function.

The cloning and sequencing of *p53* cDNAs from a variety of species has enabled detailed analyses of the structure and function of the p53 protein. There are five regions of the protein (domains I to V) which are evolutionarily highly conserved indicating a central role in p53 activity (Soussi *et al.*, 1990). Functionally, the protein can be divided into three regions; the amino-terminal domain, the central core and the carboxy-terminal domain (Figure 1). The amino-terminal domain binds to several viral and cellular proteins including the TBP (TATA box binding protein) component of the transcription factor TFIID and mdm2 (Seto *et al.*, 1992; Chen *et al.*, 1993a; Chen *et al.*, 1993b). In addition, the amino-terminal domain acts as a transcriptional activator (Farmer *et al.*, 1992). The central core of the p53 protein is able to bind DNA in a sequence-specific manner (Bargonetti *et al.*, 1991; Kern *et al.*,

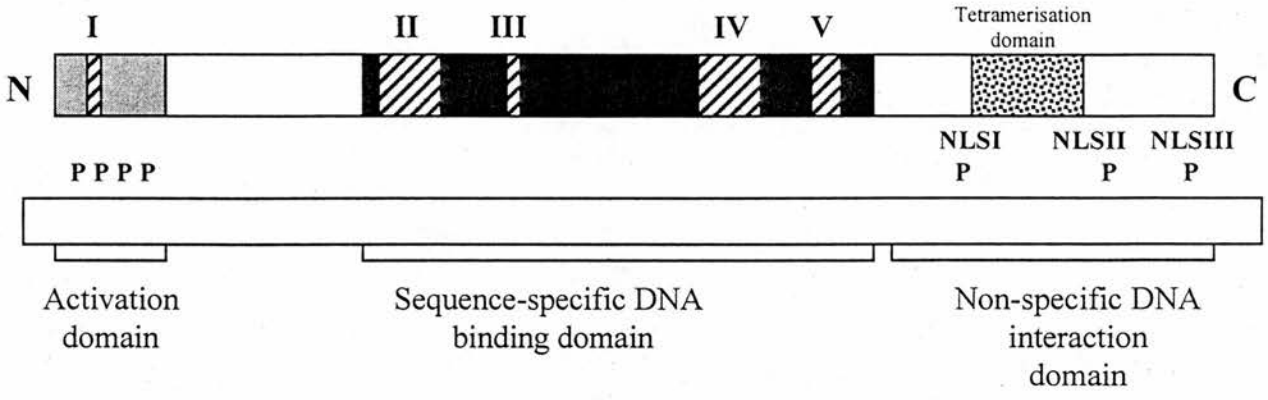


Figure 1: Structure of the human p53 protein. Hatched boxes represent evolutionarily conserved regions. N indicates the amino terminus and C the carboxyl terminus. NLS represents nuclear localisation signals and P phosphorylation sites.

1991; El-Diery *et al.*, 1992; Funk *et al.*, 1992; Zauberman *et al.*, 1993) and crystal structure analyses have revealed that the four conserved domains (II to IV) of the central core are intimately involved in this process (Cho *et al.*, 1994). The carboxy-terminal domain has multiple functions. First, p53 is known to non-specifically bind both single and double stranded DNA and catalyses DNA and RNA renaturation and DNA strand transfer (Bakalkin *et al.*, 1994; Brain and Jenkins, 1994) via its c-terminal domain (Oberosler *et al.*, 1993; Bakalkin *et al.*, 1994; Brain and Jenkins, 1994). Second, this domain has also been demonstrated to recognise one to three nucleotide long insertion/deletion mismatches (Lee *et al.*, 1995). Finally, p53 exists primarily as a tetramer (McCormick *et al.*, 1981; Friedman *et al.*, 1993) and the region of oligomerisation has been localised to the c-terminus.

There is an alternative splice variant of murine p53 (ASp53) in which the 26 carboxy terminal amino acids are substituted for 17 new amino acids (Wolf *et al.*, 1985). The physiological role of this splice variant is not yet known but it differs from the full length p53 protein (NSp53) in a number of ways. ASp53 does not possess nucleic acid reannealing activity and does not bind DNA non-specifically (Bayle *et al.*, 1995; Wu *et al.*, 1995). Unlike NSp53, ASp53 displays constitutive sequence specific binding activity and is not allosterically regulated by its c-terminus (Wu *et al.*, 1994; Bayle *et al.*, 1995; see section 1.3.2). ASp53 is able to oligomerise with p53 and the resulting complex shows the sequence specific binding properties of NSp53 (Wu *et al.*, 1994). Lastly, ASp53 and NSp53 may be expressed at different stages of the cell cycle (Kulesz-Martin *et al.*, 1994).

1.3.2 Activation of p53 following DNA damage.

Evidence for increases in p53 protein levels following DNA damage is abundant but it is now becoming clear that this is probably not the only mechanism by which p53 is activated. In most cell types, wild-type p53 is present in very small quantities and is turned over at an extremely rapid rate such that it has a half life measured in minutes. After treatment with DNA-damaging agents, levels of p53 rise rapidly via a process that is generally accepted to be post-transcriptional. This rise in p53 levels is

thought to be due to stabilisation of the protein which is normally degraded by the ubiquitin pathway (Kastan *et al.*, 1991; Chowdary *et al.*, 1994). In addition, p53 levels may also be partially controlled at the level of translation as it has been suggested that p53 can inhibit translation of its own mRNA (Mosner *et al.*, 1995). The presence of strand breaks is known to be critical for the induction of p53 (Nelson and Kastan, 1994) and even a single double-strand break may be sufficient to induce p53 stabilisation (DiLeonardo *et al.*, 1994). p53 exists in conformations that are both active and inactive for sequence specific DNA binding activity and the c-terminus has been shown to allosterically regulate this process. This regulatory function of the c-terminus appears to be confined to the last 30 amino acids which, when deleted or bound by antibody, stimulate binding activity (Hupp *et al.*, 1992; Halazonetis *et al.*, 1993; Takenaka *et al.*, 1995; Hupp *et al.*, 1995). In addition, short (up to 30 nucleotides) sections of single-stranded DNA, through interaction with the c-terminus, can stimulate sequence specific DNA binding of p53 (Jayaraman and Prives, 1995). Phosphorylation may also be involved in the induction of p53 activity. p53 is known to be phosphorylated at sites in the amino and carboxy termini, both *in vivo* and *in vitro* (reviewed by Meek, 1994). A number of different kinases have been shown to be involved in this process (reviewed by Meek, 1994). Hyperphosphorylation of p53 has been found to increase steady-state levels of p53 (Zhang *et al.*, 1994) and phosphorylation of the c-terminus has been shown to increase DNA binding activity (Hupp *et al.*, 1992; Takenaka *et al.*, 1995; Hupp *et al.*, 1995). In addition, some mutation studies have demonstrated roles for phosphorylation at particular sites in the function of p53 (Mayr *et al.*, 1995) but there is much conflicting evidence and others report little or no effect (Fuchs *et al.*, 1995). Despite these recent advances in our understanding of p53 phosphorylation, its function is still largely unresolved.

Several proteins have recently been implicated in the regulation of p53-mediated activation. These include WT1 (Wilms Tumour 1), $p33^{ING1}$, REF1 and p300 which are able to bind to p53 and enhance its transcriptional activation activity (Maheswaran *et al.*, 1995; Avantaggiati *et al.*, 1997; Jayaraman *et al.*, 1997;

Garkavtsev *et al.*, 1998). A number of these genes show considerable functional homology with p53. For example, p33 not only enhances transcriptional activation of p21 by p53, but is also able to block cell proliferation and induce apoptosis when overexpressed (Garkavtsev *et al.*, 1996; Helbing *et al.*, 1997). Another candidate tumour suppressor gene, *IRF1*, has been shown to suppress growth via the activation of p21 and is also able to interact with p53 in a synergistic fashion in the activation of this gene. In this case binding to p53 does not appear to be necessary, suggesting a different mechanism to that described for p33 (Tanaka *et al.*, 1996). Like p53, IRF1 is also able to induce apoptosis, independent of p53 activity, in response to DNA-damage (Tamura *et al.*, 1995). The recently discovered p53 homologue, p73, is able to activate the transcription of p53-responsive genes and to induce apoptosis when overexpressed (Jost *et al.*, 1997). This gene shows considerable sequence homology with p53, except in the carboxy terminal domain, but unlike p53, is expressed at low levels in all normal tissues and is not induced in response to DNA damage suggesting that it fulfils a different, and as yet unknown, purpose (Kaghad *et al.*, 1997; Jost *et al.*, 1997). The relationship between all of these genes is still poorly understood but they appear to constitute a group of genes with related, and perhaps overlapping, functions.

The precise mechanism by which p53 activates transcription is not yet clear but, like most well characterised activators, it appears to require interaction with the general transcriptional machinery. TFIID is a transcription complex containing TBP and two TBP-associated factors (TAFs), dTAFII40 and dTAFII60. p53 is known to interact with TFIID and that interaction stimulates sequence specific binding of p53 to DNA (Liu *et al.*, 1993; Chen *et al.*, 1993b). It is likely that it is p53's association with dTAFII40 and dTAFII60, rather than TBP itself which is essential for transcriptional activation (Lin *et al.*, 1994; Thut *et al.*, 1995; Lu and Levine, 1995).

1.3.3 p53-mediated transcriptional activation.

A large number of genes have been found to contain p53 binding sites and thus could be targets for p53 transcriptional activation. Confirmation of the status of many of

these candidate genes as true p53 targets is eagerly awaited but a number of good candidates have already been identified. These include *p21* (*WAF1/Cip1*), *mdm2*, *GADD45*, *cyclin G*, *Bax* and the insulin-like growth factor binding protein 3 (*IGF-BP3*). The product of the *p21* gene forms part of a quaternary complex which includes cyclins, cyclin-dependent kinases (CDKs) and the proliferating cell nuclear antigen (PCNA) (Xiong *et al.*, 1993b). At high concentrations p21 inhibits the function of CDKs, particularly those acting at the G_1 phase of the cell cycle, and thus is probably responsible, at least in part, for the p53-dependent G_1 arrest observed after DNA-damage (Gu *et al.*, 1993; Harper *et al.*, 1993; Xiong *et al.*, 1993a). Insulin-like growth factor binding protein 3 (IGFBP3) is induced following DNA-damage and inhibits signalling via insulin-like growth factor. It is thus an anti-mitogenic protein and suggests another route by which p53 might suppress growth (Buckbinder *et al.*, 1995). The *mdm2* gene encodes a protein which binds to p53 and results in repression of its transcriptional transactivation activity and enhanced proteosome-dependent degradation (Momand *et al.*, 1992; Oliner *et al.*, 1993; Haupt *et al.*, 1997; Kubbutat *et al.*, 1997). The *mdm2* gene is itself a target of p53 activation implying an autoregulatory feed-back loop between p53 and mdm2 (Barak *et al.*, 1993; Perry *et al.*, 1993; Wu *et al.*, 1993). The *cyclin G* gene is induced, in a p53-dependent manner, subsequent to DNA-damage but its function is not yet clear (Okamoto and Beach, 1994; Zauberman *et al.*, 1995). The product of the *Bax* gene (see section 1.2.4.1) is an inducer of apoptosis which is activated, by p53, following DNA-damage and provides a possible mechanism for p53-mediated apoptosis (Zhan *et al.*, 1994; Miyashita and Reed, 1995).

1.3.4 p53-mediated transcriptional repression.

The mechanism of p53-mediated transcriptional repression is not well understood. p53 has been reported to repress the transcription of proteins driven by a broad range of promoter structures and this repression activity has been proposed to play a role in p53-mediated apoptosis. Both the amino- and carboxy-terminals have been implicated in repression activity (Shiio *et al.*, 1992; Sang *et al.*, 1994; Subler *et al.*, 1994; Shaulian *et al.*, 1995). It has been reported that only those promoters

containing TATA boxes and not containing initiator elements are inhibited by p53 (Mack *et al.*, 1993). Deletion studies indicate that binding to both TBP and TAFs may be required for transcriptional repression (Sabbatini *et al.*, 1995; Ko and Prives, 1996). It has been suggested that p53-mediated transcriptional repression may be the result of the interaction and functional sequestration of components of the transcriptional apparatus by p53 (Ko and Prives, 1996).

1.3.5 p53, cell cycle arrest and apoptosis.

Following exposure to DNA-damaging agents, levels of p53 rise rapidly and cells may either enter apoptosis or undergo a G_1 arrest (Fritsche *et al.* 1993). Both of these processes have been shown to be p53-dependent in a variety of cell types (Kastan *et al.*, 1991; Kuerbitz *et al.*, 1992; Yonish-Rouach *et al.*, 1991; Clarke *et al.*, 1993; Lowe *et al.*, 1993; Clarke *et al.*, 1994; Morgenbesser *et al.*, 1994; Lu and Lane, 1993; Mortensen *et al.*, 1992).

The *p21* gene plays a critical role in p53-mediated G_1 arrest. Subsequent to irradiation, up-regulation of p21 can inhibit both cyclin E/CDK2 and cyclin A/CDK2, resulting in the accumulation of hypophosphorylated RB and G_1 arrest (Dulic *et al.*, 1994; Demers *et al.*, 1994; Slebos *et al.*, 1994). Studies showing that p21 overexpression can lead to G_1 arrest and that *p21*-null cells show defects in radiation induced cell cycle arrest further support the crucial role of p21 in p53-dependent G_1 arrest, but the potential involvement of other p53 targets, perhaps p53-binding protein 2 (53BP2, Naumovski and Cleary, 1996), cannot be excluded (Harper *et al.*, 1995; Brugarolas *et al.*, 1995; Deng *et al.*, 1995). In addition, recent evidence has shown that p53-independent pathways to p21-mediated G_1 arrest, involving the transcription factor IRF1, also exist (Tanaka *et al.*, 1996). Originally it was believed that the role of p53-mediated G_1 arrest was to allow time for DNA-repair before continuation of the cell cycle (Kastan *et al.*, 1991) however, it is now clear that G_1 arrest is not always a transitory response; following DNA-damage, human fibroblasts enter a prolonged and irreversible G_1 arrest (DiLeonardo *et al.*, 1994). p53 has also been implicated in G_2 arrest (Ryan *et al.*, 1993; Kulesz-Martin *et al.*, 1994; Agarwal

et al., 1995; Aloni-Grinstein *et al.*, 1995; Guillouf *et al.*, 1995; Powell *et al.*, 1995; Stewert *et al.*, 1995) and recent evidence has shown p53 to be involved in a mitotic spindle checkpoint. When exposed to spindle inhibitors, *p53*-null murine fibroblasts, unlike their wild-type counterparts, do not arrest but undergo multiple rounds of DNA synthesis, without the appropriate chromosome segregation, resulting in the formation of tetraploid and octaploid cells (Cross *et al.*, 1995).

p53 has been shown to mediate apoptosis both when over-expressed in cell lines lacking endogenous, wild-type p53 (Yonish-Rouach *et al.*, 1991) and under physiologically relevant conditions (Clarke *et al.*, 1993; Lowe *et al.*, 1993; Clarke *et al.*, 1994; Merrit *et al.*, 1994). p53-dependent apoptosis has been observed in a wide variety of cell types (reviewed by Oren, 1994) and in response to a broad range of stimuli which include DNA-damaging agents, hypoxia, heat shock, survival or growth factor withdrawal and some cytokines (Graeber *et al.*, 1994; Canman *et al.*, 1995; Eizenberg *et al.*, 1995). However, not all pathways to apoptosis are p53-dependent; for example dexamethasone treatment of thymocytes results in p53-independent apoptosis (Clarke *et al.*, 1993). The p53-dependent apoptotic response shows a gene-dose effect, with cells having only one functional copy of p53 exhibiting intermediate levels of apoptosis between that shown by wild-type and *p53*-null cells (Clarke *et al.*, 1993; Lowe *et al.*, 1993). However, there is some evidence to suggest that, at least in some cell types, the absence of p53 does not result in reduced sensitivity to apoptosis but merely changes the kinetics of its induction (Xia *et al.*, 1995). The mechanism of p53 induced apoptosis is still poorly understood and its resolution has been complicated by the fact that the majority of studies have used cell types either over-expressing p53 or with genetic backgrounds in which the normal function of p53 may be disrupted. Expression of the survival factor, *BCL2*, can inhibit p53-mediated apoptosis (Debbas and White, 1993; Chiou *et al.*, 1994; see section 1.2.4.1) as can a variety of growth factors (Johnson *et al.*, 1993; Yonish-Rouach *et al.*, 1993; Gottlieb *et al.*, 1994; Canman *et al.*, 1995). Although the ability of p53 to function as a transcriptional activator is necessary for its mediation of G_1 arrest, several studies have indicated that this function may not be required, in some cell types, for the induction of apoptosis (Caelles *et al.*, 1994; Wagner at al., 1994;

Haupt *et al.*, 1996). It seems probable that there are at least two distinct mechanisms via which p53 can mediate apoptosis, the first requiring transcriptional activation, perhaps of BAX (see sections 1.2.4.1 and 1.3.2), and the second not. Aside from its function as a transcriptional repressor or activator, p53 also interacts directly with a number of cellular proteins and it may be these interactions which are relevant to its role in apoptosis. There is evidence that binding of the TFIIH protein complex is critical for at least one pathway of p53-dependent apoptosis that does not require p53-mediated transcriptional activation (Wang *et al.*, 1996c).

When exposed to DNA-damaging agents, cells may respond in one of two ways; cell cycle arrest or apoptosis. What determines which of these two processes is the outcome of increased levels of p53 expression is still unclear but cell type appears to be an important determining factor (Midgley *et al.*, 1995; Haupt *et al.*, 1996). There is some data to suggest that, at least in some circumstances, p53 simultaneously signals growth arrest and apoptosis and that, without downstream inhibition, apoptosis is the normal outcome. For example, abrogation of p53-mediated apoptosis, by BCL2, in a myeloid leukaemia cell line revealed a p53-dependent G_1 arrest (Guillouf *et al.*, 1995). There is also substantial evidence indicating that conflicting signals for proliferation and growth arrest result in the induction of apoptosis. Thus, over-expression of *c-myc* in growth arrested murine fibroblasts results in apoptosis but in the absence of p53 merely results in cell cycle re-entry (Evan *et al.*, 1992; Hermeking and Eick, 1994). Similar links between deregulation of proliferation signals and apoptosis have been demonstrated *in vivo* (Morgenbesser *et al.*, 1994).

1.3.6 p53 and karyotypic instability.

It has been suggested that loss of *p53* results in karyotypic instability and evidence supporting this hypothesis comes from a number of sources. Firstly, fibroblasts derived from Li-Fraumeni patients (a familial cancer syndrome in which patients develop a wide spectrum of primary tumours at an early age as a result of a germline mutation in one allele of the *p53* gene) and grown *in vitro* commonly develop

chromosomal abnormalities (Bischoff *et al.*, 1990). Similarly, the majority (67%) of murine embryo fibroblasts derived from *p53* -/- mice have been found to be aneuploid by passage three and this proportion rises to 95% by passage 25 (Harvey *et al.*, 1993; Tsukada *et al.*, 1993). Moreover, loss of *p53* is often associated with aneuploidy (Meling *et al.*, 1993; Purdie *et al.*, 1994) and gene amplification (Yin *et al.*, 1992; Livingstone *et al.* 1992; Donehower *et al.*, 1995) in a variety of tumour types. Secondly, *in vivo* studies on a variety of cell types from *p53*-null mice show an elevated frequency of chromosomal abnormalities, frequent gene amplification and abnormal centrosome amplification (Bouffler *et al.*, 1995; Fukasawa *et al.*, 1997). Recent evidence has shown p53 to be involved in a mitotic spindle checkpoint and when exposed to spindle inhibitors, loss of this checkpoint in *p53*-null cells results in the accumulation of tetraploid and octaploid cells. It may be that loss of this checkpoint is the causative factor in the observed *in vivo* increases in aneuploidy of *p53*-null cells (Cross *et al.*, 1995; see section 1.3.5). However, the majority of *p53*-null mice survive to birth (Donehower *et al.*, 1992), although they are subject to an increased frequency of developmental abnormalities (Sah *et al.*, 1995; Armstrong *et al.*, 1995), suggesting that there are other p53-independent mechanisms for the maintenance of karyotypic stability.

1.3.7 The role of p53 in DNA repair.

Several authors have suggested that p53 may be required for NER but evidence for this is conflicting. Both p21 and GADD45, which are induced by p53 following DNA damage, bind to PCNA which is required for NER and DNA synthesis (Shivji *et al.* 1992; Prelich *et al.* 1987). Binding of p21 to PCNA inhibits DNA replication but not NER (Waga *et al.*, 1994; Li *et al.*, 1994) whilst the function of GADD45/PCNA binding is still unclear (Smith *et al.*, 1994; Kazantsev *et al.*, 1995). In addition, p53 has been shown to bind to a number of proteins known to be involved in DNA repair and replication. These include replication protein A (RPA), several components of the TFIIH transcription-repair factor (p62 and the helicases XPD (xeroderma pigmentosum complementation group D, also known as ERCC2/Rad3) and XPB (xeroderma pigmentosum complementation group B, also

known as ERCC3) and CSB (Cockaynes syndrome complementation group B, also known as ERCC6) (Dutta *et al.*, 1993; He *et al.*, 1993; Li and Botchan 1993; Wang *et al.*, 1994; Xiao *et al.*, 1994; Wang *et al.*, 1995; Leveillard *et al.*, 1996). p53 is also known to non-specifically bind both single and double stranded DNA and catalyses DNA renaturation and strand transfer suggesting it may play a more direct role in DNA repair (Bakalkin *et al.*, 1994; Brain and Jenkins, 1994). Interactions of RPA and TFIIH with p53 are not, however, unique and may relate to the function of p53 as a transcriptional transactivation protein (He *et al.*, 1993; Li and Botchan, 1993; Xiao *et al.*, 1994; Tong *et al.*, 1995). A role for p53 in DNA damage recognition has also been suggested by the formation of stable complexes between the p53 protein and insertion/deletion mismatches and irradiated DNA (Lee *et al.*, 1995; Reed *et al.*, 1995). Disruption of normal p53 function in RKO cells has been shown to result in reduced repair of UVC induced damage (Smith *et al.*, 1995) but perhaps the most convincing evidence for the involvement of p53 in NER is the reduced repair capacity of Li-Fraumeni syndrome cells which are heterozygous for *p53* mutation (Ford and Hanawalt, 1995; Wang *et al.*, 1995). However, *p53*-null mouse fibroblasts display normal rates of repair (Ishizaki *et al.*, 1994; Sands *et al.*, 1995). Similarly, p53-null and wild-type embryonic stem (ES) cells show a similar overall capacity to repair lesions in a UVC damaged reporter plasmid although, unexpectedly, the rate of repair was faster in *p53*-null cells (Prost *et al.*, 1998).

It has been predicted that a *p53*-null environment would result in an increased mutation frequency (Lane, 1992). *In vivo* studies in *p53*-null mice transgenic for a *lacI* (galactosidase inhibitor) lambda shuttle vector, termed Big Blue, suggest that this is not the case as no increase in spontaneous mutation frequency was observed (Nishino *et al.*, 1995; Sands *et al.*, 1995). However, the assay used in these studies is only able to detect small mutations and not large deletions or insertions (Nishino *et al.*, 1995). The situation is less clear following high levels of DNA damage. *In vitro* studies on Big Blue embryonic fibroblasts treated with 4-nitroquinoline 1-oxide (4NQO), which induces single strand breaks, again showed no greater increase in mutation frequency in *p53*-null cells (Sands *et al.*, 1995) but an *in vivo* analysis of

mutation frequency at the *Dlb-1* (Dolichos biflorus 1) locus in *p53*-null mice after γ-irradiation showed p53 dependence at high doses (6 Gy) (Clarke, *et al.*, 1997).

1.3.8 p53 and differentiation.

It has been suggested that p53 plays a role in the regulation of differentiation of several cell types based on observations correlating increased p53 expression with the expression of cellular differentiation markers. Over-expression of *p53* in pre-B cells results in a differentiated phenotype and the expression of immunoglobulin μ heavy chain (Shaulsky *et al.*, 1991). Similarly, introduction of wild-type p53 in HL-60 cells, K562 cells, squamous carcinoma cells or Friend virus transformed erythroleukemic cells results in partial differentiation (Feinstein *et al.*, 1992; Brenner *et al.*, 1993; Soddu *et al.*, 1994; Bannerjee *et al.*, 1995; Ehinger *et al.*, 1995). In addition, dominant negative mutants of p53 are able to suppress differentiation in some untransformed cell types (Soddu *et al.*, 1996). It is important to note that p53 also results in perturbation of cell cycle progression in these studies and thus it is possible that the expression of differentiation markers is the result of proliferation inhibition and not a direct consequence of p53 expression. Moreover, the observation that differentiation of all of these cell types apparently proceeds normally in *p53*-null mice indicates that p53 is not essential to this process.

1.4 Aims.

In summary, apoptosis is a controlled process of cell death that can be triggered by both cellular injury and physiological stimuli. Whatever the triggering stimulus, signalling pathways feed into a common terminal execution pathway. Cellular injury stimuli include cell membrane injury, DNA damage and mitochondrial injury. Physiological stimuli may induce apoptosis via either non-transcriptional pathways, by binding of CD95 ligand for example, or by transcriptional activation of genes like *reaper* and *E2F1*. It is also clear that these apoptosis signalling pathways are able to interact with each other.

There is plentiful evidence that abnormalities in these pathways lead to disease. Examples include autoimmune lymphoproliferative disorders caused by mutations in

CD95; profound and lethal developmental disorders of the nervous system in caspase 3 deficient mice and developmental and immunological disorders of mice deficient in BCL2 and BCLX. Historically, cancer has been viewed as the result of abnormal cellular proliferation but it is now widely recognised that this is an oversimplification. Carcinogenesis is the result of failure of cellular homeostasis, a finely tuned balance between both cellular proliferation and cell death. Cancer is a multi-step process typically characterised by the accumulation of genetic lesions in both of these processes and a number of tumour suppressors and oncogenes are now known to be involved in the regulation of cell death (reviewed by Lyons and Clarke, 1997). As previously discussed, the BCL2 gene was first identified by its involvement in B-cell lymphoma and p53 is also one of the most commonly mutated genes in human cancers (Hollstein *et al.*, 1994). In addition, a number of oncogenic viruses, including HPV and Epstein-Barr virus (EBV), have developed strategies for blocking apoptosis, emphasising the importance of this process in carcinogenesis (reviewed by Young *et al.*, 1997). This thesis aims to address some of the remaining questions on the role of p53 in these processes. In particular, does the high frequency of p53 mutation in cancer reflect the fact that absence of p53 function leads to survival of cells with undetected or inappropriately repaired DNA-damage?

The aims of this study were to investigate the immediate and long term consequences of a *p53*-null environment on cell survival and the acquisition of mutations subsequent to treatment with DNA-damaging agents.

The majority of studies of this type have utilised either short-term primary cultures or immortalised cell lines and thus data on stem cells, which are perhaps more relevant to the development of neoplasia, are lacking. Because of the difficulties of working on stem cells *in vivo*, it was decided that these relationships would be examined *in vitro* using ES cells. ES cells are derived directly from the early embryo and are capable of continuous, undifferentiated growth *in vitro*. ES cells are also pluripotent and when introduced into an early embryo, participate fully in normal embryonic development giving rise to chimaeric mice. It would be expected that damage to the

DNA of ES cells would have far reaching repercussions for the organism as ES cells ultimately contribute to all cell types. Consequently, it has been argued that mechanisms for the resolution of DNA damage, whether by repair or death, would be particularly effective in these cells.

In order to achieve the aims of this project, it was necessary to obtain a *p53*-null ES cell line. This was derived from an existing cell line bearing a heterozygous, targeted deletion of the *p53* gene. Comparisons between wild-type, heterozygous and *p53*-null cell lines were subsequently used to characterise the cellular response to DNA-damage. Specifically, it was intended that the role of p53 in immediate and long term survival and the acquisition of mutations, subsequent to DNA-damage, should be examined. To address these questions, several agents have been used to induce DNA-damage in ES cells and the effects of a *p53*-null environment on cell survival have been assessed. As survival in the short term does not necessarily correlate with continued proliferative capacity, both immediate survival, measured in terms of apoptosis, and clonogenic survival have been investigated. In addition, the temporal correlation between apoptosis and the expression of p53 has been examined. As previously discussed, p53 has been implicated in DNA repair and it has been predicted that its loss would result in an increased mutation frequency. In this project, the role of p53 in the acquisition of mutations at the hypoxanthine phosphoribosyl transferase (*Hprt*) locus has been assessed subsequent to two different types of DNA-damage; UVC-irradiation and γ-irradiation. These two DNA-damage inducing agents result in quite distinct spectrums of DNA damage (see section 2.2) and employ different repair pathways.

CHAPTER TWO

MATERIALS AND METHODS

Where not given in the text, suppliers of reagents are listed in appendix II. Deionised distilled water (DDW) was prepared using the Elga Maxima system (Elga).

2.1 Cell Culture.

2.1.1 Cell lines.

All murine embryonic stem cell lines were derived from mouse strain 129/Ola. Wild-type cell lines used included E14 (Hooper *et al.*, 1987), CGR8 (Mountford *et al.*, 1994) and HM1, a spontaneous *HPRT* mutant derived from E14 (Magin *et al.*, 1992). The *p53*-null cell lines (R72D5, R72D27 and R72D54) were derived from the heterozygous targeted cell line R72 (see section 2.1.6; Purdie, 1994; Clarke *et al.*, 1993). R72 was derived from the E14 wild-type ES cell line. R721, R722 and R723 are subclones of R72.

2.1.2 Maintenance of stock ES cell cultures.

Cells were grown at 37°C and under 5% CO_2 in CM5-5 cell culture medium (see appendix I). The medium was replaced when a change in the colour of the medium from red to amber indicated that it was becoming exhausted (usually daily). All constituents of the medium and other tissue culture reagents were batch tested to ensure that they were able to support ES cell growth. During batch testing, a single new component was tested in medium otherwise derived from constituents that had previously batch tested satisfactorily. A component was found to be acceptable if it gave homogenous stem cell morphology, over a period of at least 10 days, to a similar or greater level than previously tested medium and resulted in cloning efficiencies between 10 and 30 % (see section 2.1.8 for clonogenic assay). In order

to minimise all potential extraneous sources of variation and facilitate comparisons between data points, medium and tissue culture reagents for use in any single experiment were all derived from constituents from a single batch. Cells were sub-cultured when sub-confluent and were routinely split 1 in 10 every three days

2.1.3 Production of recombinant LIF.

Cos-7 cells (Gluzman, 1981) were transfected with the murine leukaemic inhibitory factor (LIF) expression vector pC10-6R (Smith *et al.*, 1988). Cells were trypsinized and resuspended in phosphate buffered saline (PBS) at a concentration of $1x10^8$/ml. A 0.8ml aliquot of this cell suspension was electroporated with 120μg of LIF plasmid at 200V and 500μFd. Cells were left for 10 minutes and then plated out in a 10cm dish with 20ml of medium. The following day, the medium was removed and replaced with 25ml of fresh medium. After three days, the conditioned medium was collected and a further 25ml of medium added to the plates. This was removed after two days, combined with the previous batch of conditioned medium and stored at -70°C. The conditioned medium was tested for biological activity by monitoring the morphology of ES cell cultures maintained in CM5-5 medium supplemented with differing dilutions of the LIF medium. An excess of LIF did not appear to have any adverse effects on either ES cell growth or differentiation state. Therefore, in subsequent experiments LIF medium was used at double the lowest concentration that gave homogenous stem cell morphology (usually around 1:1000).

2.1.4 Trypsinisation of cell cultures.

Prior to trypsinisation, fresh flasks were prepared by incubating for at least 15 minutes with 0.1% gelatin (see appendix I). Cultures were washed with PBS and then incubated at 37°C with sufficient TVP (phosphate buffered trypsin/versine, see appendix I) to cover the bottom surface of the flask. When cells had disaggregated and could be dislodged from the bottom surface of the flask by gentle tapping, the trypsin was neutralised by the addition of two volumes of fresh medium, and cells transferred to fresh flasks. Cultures were left for at least two hours to allow cells to adhere to the floor of the flask and then the medium was removed and replaced.

2.1.5 Production of frozen cell stocks.

Large quantities of frozen cell stocks were prepared for each cell line used in order that all experiments could be performed with cells of a similarly low passage. Sub-confluent cells were trypsinised as above and centrifuged at 100 x g for five minutes. The growth medium was removed, and cells were resuspended at approximately 1 x 10^7/ml in CM5-5 medium supplemented with 10% foetal calf serum (FCS) and 10% dimethyl sulphoxide (DMSO). One ml aliquots of cells were cooled to -70°C at approximately 1°C/min and then transferred to long term storage in liquid nitrogen vapour. To thaw, cells were placed at 37°C, the suspension diluted with 5ml of medium and centrifuged at 100 x g for five minutes. The supernatant was removed and cells resuspended in 8ml of fresh medium, which was then used to inoculate a 25cm^2 flask. Cultures were left for at least two hours to allow cells to adhere to the floor of the flask and then the medium was removed and replaced.

2.1.6 The derivation of *p53*-null ES cell lines by high G418 selection.

The antibiotic Geneticin (G418) is toxic to mammalian cells but resistance can be conferred by the expression of the bacterial neomycin resistance gene (*neo*). The high G418 selection technique is based on the principle that homozygous cells are occasionally spontaneously produced from heterozygous cells in culture and that, if heterozygotes bear a selectable marker closely linked to the gene of interest, such homozygotes can be selected by the use of selective media at a concentration that is normally toxic to heterozygote cells (Mortensen *et al.*, 1992). The cell line R72 is heterozygous for *p53* and bears a *neo* resistance cassette in the targeted allele and was thus thought to be suitable for this selection protocol.

R72 ES cells were plated on 10 10cm plates at 5x10^6. The following day, medium was removed and replaced with medium containing 0.25, 0.375, 0.5, 0.75 and 1 mg/ml active G418 (two plates at each dose). Cultures were maintained in this selective medium for 10 days. Only those cultures maintained in 1mg/ml G418 gave rise to clones, the remainder of the plates being confluent or sub-confluent. Clones on the 1mg/ml plates were densely packed (around 1000 to 2000 colonies per plate)

making it very difficult to isolate single clones and so a second series of plates were set up, again using R72 ES cells, for selection in G418 concentrations of 1, 1.5 and 2mg/ml. Those plates selected in 2mg/ml G418 gave rise to fewer colonies (400 to 800 clones per plate) than those selected in 1mg/ml but were still fairly densely packed. Clones were isolated from both the 1 and 2 mg/ml selection regimes.

2.1.7 Isolation of clones.

The tips of glass Pasteur pipettes were heated in a Bunsen flame until the glass became molten and then stretched. Careful control of the heating and stretching process allowed the production of pipettes of varying bore width. Clones were picked in a still air hood, under sterile conditions. Using a binocular dissecting microscope (Wessex WZ1), individual clones were gently teased off the surface of the plate with a very fine bore pipette. These clones were removed from the plate, with as little medium as possible, using a wide bore pipette and placed in a second sterile plate. Two drops of TVP were added to the clone and the plate placed at 37°C for approximately five minutes to allow disaggregation of the clone. The clone was passed through a fine Pasteur pipette (three to five cell widths wide) several times to break apart the larger cell clumps and then placed in a single well of a 24 well plate with 0.5 ml of medium. It was usually necessary to trypsinise clones and transfer them to new wells at least once before a sub-confluent culture was obtained in order to prevent excessive cell death and differentiation due to cell clumping. When sub-confluent, clones were trypsinised, half of the cells frozen down and the remainder pelleted and frozen at -20°C for DNA extraction and analysis. DNA analysis revealed that some clones were not of monoclonal origin and thus clones of potential interest were thawed, serially diluted (at concentrations ranging between 2 and 25 cells per well) and plated in 24 well plates. After careful observation of the plates to confirm monoclonal origin, a second clone was selected and expanded for future analysis.

2.1.8 Clonogenic survival Assays.

Because of the anticipated reduction in clonogenic survival subsequent to DNA damage, it is necessary to plate out treated cells at a higher cell density than would normally be used to assess plating efficiency. It is known that the plating efficiency of mass cultures is much higher than that achieved in very sparse cultures and this raises concerns over the suitability of such an assay when used to compare cells seeded at a wide variety of cell densities. To address this issue it is necessary to assess the linearity of the relationship between plating density and clonogenic survival over as wide a range of plating densities as possible.

Cells were trypsinised, using standard protocols, and the trypsin neutralised with two volumes of cell culture medium. An aliquot of cell suspension was removed and the cell density counted using a haemocytometer. The suspension was also examined to ensure it contained only single cells and not clumps. Cells were plated out, in triplicate, at densities of 10^2, 2.5×10^2, 5×10^2, 7.5×10^2, 10^3, 2.5×10^3, 5×10^3, 7.5×10^3 and 10^4 cells per 10cm plate. Plates were maintained for 10 days and fed as appropriate. After 10 days, the medium was removed from the plates and the cells fixed in 70% bench alcohol (74°P). Plates were then stained in 5% Giemsa (Sigma), and colonies counted. Morphology of the clones was assessed microscopically and only those clones with predominantly ES cell morphology were counted (some differentiation at the periphery of the colony was permitted). The results indicated that over the plating density range of 10^2 to 2.5×10^3, the relationship between plating density and clonogenic survival was linear (Figure 2). At the higher plating densities of 10^4, 7.5×10^3 and, to a lesser extent, 5.0×10^3 clones were very densely packed (up to 1338 clones per 10cm plate) and thus the likelihood of an apparently single clone actually being derived from two clones in close proximity was relatively high. Consequently it is likely that cloning efficiency at these densities has been underestimated and this would account for the slightly lower survival estimates obtained. It was therefore decided that, provided the number of surviving clones lay within the observed linear range, this assay could be used to assess the loss of clonogenic potential resulting from treatment with DNA-damaging agents.

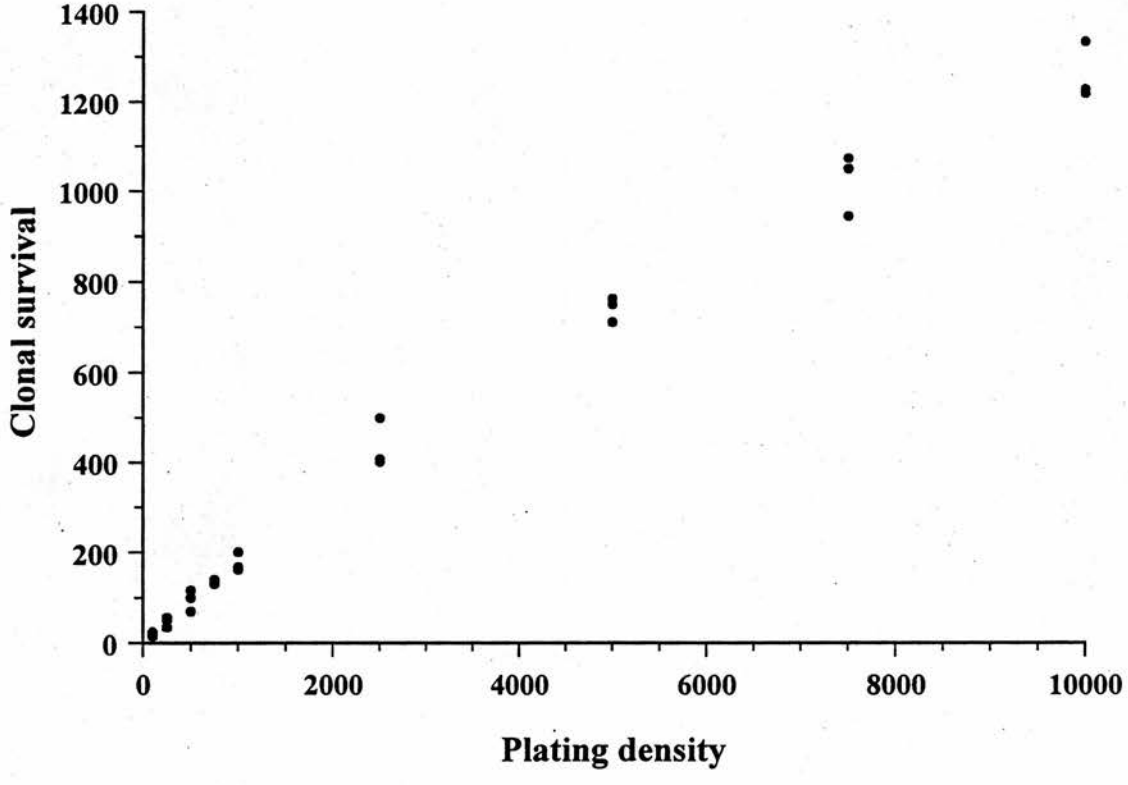


Figure 2: Scatter plot showing the clonal survival of ES cells (E14) plated at different densities to illustrate the relationship between plating density and survival.

After treatment with DNA-damaging agents, cells were trypsinised and counted using a haemocytometer. Control plates were prepared with 10^3 cells per 10cm plate and a range of cell densities from 10^3 to 10^6 prepared for treated cells. All plates were prepared in triplicates or quadruplicates.

2.1.9 *Hprt* mutation assays.

Hypoxanthine phosphoribosyl transferase (HPRT) is a vital component of the salvage pathways of purine synthesis and catalyses the regeneration of inosine monophosphate (IMP) and guanine monophosphate (GMP) from hypoxanthine and guanine respectively (Williamson et al., 1992). In its absence, cells are forced to use the *de novo* purine synthesis pathways at a much greater energy cost. It is possible to select both for and against *Hprt* mutants by the use of growth medium containing either 6 amino-6-mercaptopurine (6-TG) or hypoxanthine, aminopterin and thymidine (HAT) (Hooper, 1985). If 6-TG is included in the growth medium, cells possessing active HPRT will metabolise this purine analogue, resulting in cell death. Thus only *Hprt* mutants can survive 6-TG selection. Similarly, aminopterin blocks the *de novo* synthesis pathway and thus HAT medium is toxic to cells not having an active *Hprt* gene.

Before treatment with DNA-damaging agents, cultures were maintained in HAT medium (see appendix I) for five days to remove any existing *Hprt* mutants from the cultures. Cultures were then irradiated with either UVC or γ-irradiation and maintained in normal medium for seven days to allow for the depletion of any pre-existing HPRT in the cells. At no point were cell numbers allowed to fall below the number originally irradiated. Cells were then plated out at a density of 5×10^5, on 10cm plates, with 6-TG medium (see appendix I). Twenty to 60 plates were used for each dose, depending on the expected mutation frequency. Control plates with 10^3 cells per plate (in quadruplicate) were also prepared and maintained on normal growth medium. Plates were fixed and stained after ten days.

2.2 The induction of DNA damage and subsequent analyses.

It was necessary to find a suitable DNA-damaging agent that could be used to investigate the role of p53 in cell death and the acquisition of mutations. Four DNA-damaging agents were considered. These included UVC-radiation, γ-radiation and the topoisomerase I and II inhibitors, camptothecin and etoposide. The mechanism of induction of DNA damage differs for these agents and each results in a slightly different spectrum of lesions. The most commonly formed lesions subsequent to UVC-irradiation, are cyclobutane pyrimidine dimers and 6-4 photoproducts (Lippke et al., 1981; Setlow and Carrier, 1966). These lesions are known to block DNA synthesis and are repaired by the NER pathway, thus creating a transient single strand break (ssb). γ -irradiation causes a variety of DNA lesions, predominantly ssbs and base and sugar damage, but double strand breaks (dsb) appear to be the most biologically significant lesion and it has been suggested that a single unrepaired dsb is lethal (Frankenberg et al., 1981; Frankenberg-Schwager et al., 1982; Radford, 1985). Topoisomerase inhibitors bind to and stabilise the cleavable complex formed by the binding of topoisomerases to DNA. This prevents re-ligation of the DNA after cleavage and thus generates either double (etoposide) or single (camptothecin) strand breaks (Long et al., 1984; Nelson et al., 1984; Hsiang et al., 1985). Hence, all four stimuli induce strand breaks, although indirectly in the case of UVC, and consequently might be expected to induce the expression of p53.

2.2.1 UVC-irradiation.

UVC-irradiation was performed at 254 nm using a Spectrolinker XL-1500 (Spectronics Corp.). Cells were grown in monolayers, and all medium was removed from plates prior to irradiation.

The shape of the first series of clonogenic survival curves obtained after UVC-irradiation (Figure 3) was atypical. There was substantial but variable cell killing at doses as low as 1 and 2 Jm^{-2}, and at higher doses the curve flattened out with little additional killing. This suggested possible methodological problems. Inaccuracies in delivery of the UVC dose were found to be the cause. To address this problem, the

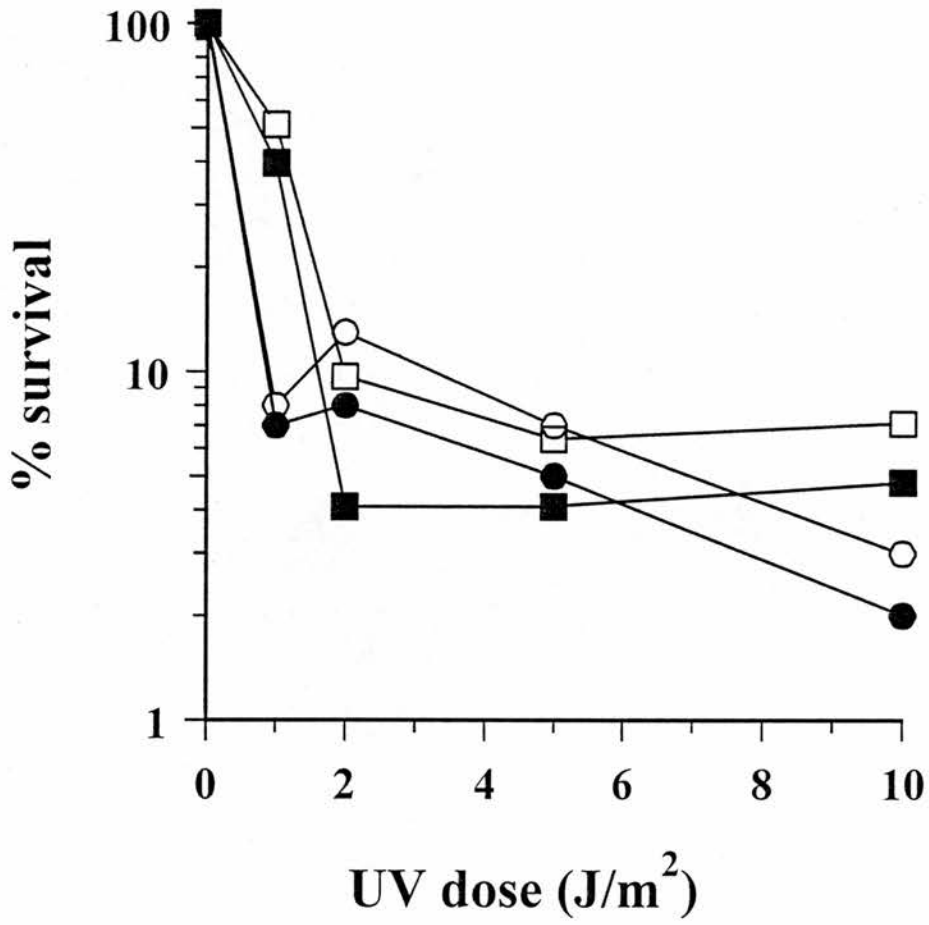


Figure 3: Clonogenic survival of wild-type (E14, solid symbols) and *p53* null (R72D5, open symbols) cells following UVC-irradiation. Results shown are from two separate experiments and cells treated at the same time are depicted with the same symbol type. Each data point represents the mean of four replicates.

UVC-irradiation cabinet was tested in collaboration with Dr S. Pye (Medical Physics, Western General Hospital, Edinburgh). A spectroradiometer (Macam SRE100, 2nm bandwidth, double monochromater) and a filtered photodiode detector (Macam UVC103 (254nm)) were used to achieve this. Three Spectrolinker XL-1500 (Spectronics Corp.) cabinets were tested, all of which were found to be inaccurate. Two of the machines tested, including the one used in our experiments, were found to have highly inaccurate internal sensors. The internal sensor's measurement of irradience, used by the machine to calculate dose delivery times, varied by a factor of up to five from the true irradience at the centre of the floor of the cabinet (Figure 4). In addition, in all machines the irradience was not uniform across the floor of the delivery chamber. The highest irradience was in the centre of the chamber (5.58 $mWcm^{-2}$), and this fell to almost half (2.92 $mWcm^{-2}$) at the edges. By using only the central region (a square 20cm x 20cm) of the chamber it was possible to restrict this variation to less than 15%. Finally, it was necessary to take into account the fact that the sides of the plates in which cells were irradiated (which are practically UVC opaque) would shadow cells at the edge of the plate, thus reducing the dose delivered. A 5mm diameter sensor was used and the average dose over the outer 5mm was found to be 70% of that in the centre. Thus errors caused by this shadow effect are likely to be small.

Although the Spectrolinker internal sensor was inaccurate, the timing mechanism was found to perform correctly, and thus accurately measured doses could be delivered. To ensure that the irradience was consistent from one dose to the next, the machine was pre-warmed for two minutes prior to irradiation. However, because the doses used were small, it was necessary to use an attenuator to achieve an appropriate irradience. Suitable attenuators were made from multiple layers of thin, semi-opaque plastic and were used in all subsequent experiments. These were suspended 4cm above the cell cultures and extended to the walls of the cabinet so as to prevent light leakage. The transmission, at 254nm, of the attenuators was measured both before and after the series of experiments and did not differ.

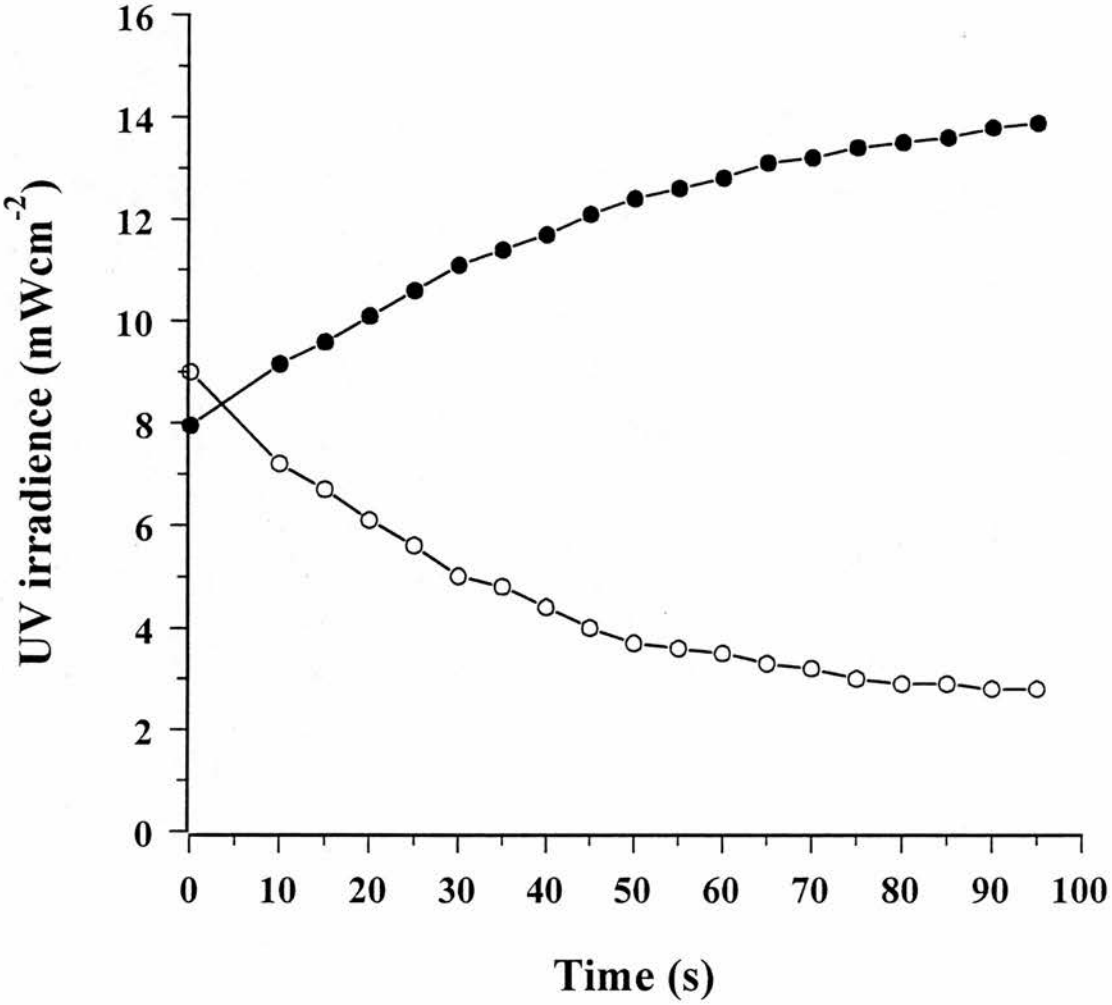


Figure 4: Comparison of the irradience measurements obtained from the internal sensor of the irradiation cabinet (open symbols) and spectroradiometer (closed symbols).

significantly. An attenuator consisting of two layers of plastic was found to have a transmission of $5.6x10^{-3}$ (+/- 5%) and a three layered attenuator $4.6x10^{-4}$ (+/- 5%).

Because of time constraints, it was not possible to repeat all of the experiments in which UVC-irradiation had been used. A separate experiment was thus carried out in which each of the irradiation protocols originally used was duplicated at least five times, and the actual UVC dose delivered was measured on each occasion. The standard deviation of the dose delivered for each irradiation protocol was less than 5%. The uncertainty in the absolute irradience measures was +/- 10%.

2.2.2 γ-irradiation.

Cells were γ-irradiated in tissue culture flasks at 20.7Gy per minute using a Cs^{137} source. Cells were transported to and from the irradiation source, a journey of between 25 and 35 minutes, in an insulated container.

2.2.3 Drug treatments.

Stock solutions of Etoposide (Sigma) and Camptothecin (Sigma) were prepared in DMSO under sterile conditions. Working strength dilutions were then made in normal growth media. In high enough concentrations DMSO is itself toxic. Concentrations were therefore kept below toxic levels and as low as possible. In addition, controls containing the same percentage of DMSO as that in the drug dilutions were included in each experiment.

2.2.4 Preparation of cells for acridine orange counting.

Acridine orange is a metachromatic fluorochrome that differentially stains double-stranded versus denatured DNA. The dye is an intercalating agent that, on excitation with UVC light, emits green light when bound to double-stranded DNA and red light when bound to denatured DNA. The nucleus of a stained cell thus appears green under UVC light whilst the cytoplasm stains red. This staining allows for the examination of nuclear morphology and hence the detection of cells undergoing apoptosis.

At selected time points after irradiation or drug treatment, cells were trypsinised and centrifuged, together with the medium and PBS from the pre-trypsinisation wash. The supernatant was discarded, cells resuspended in PBS and two volumes of ice cold fix (90% ethanol, 10% formaldehyde) added. Cell suspensions were stored at 4°C until counted. Before counting, cells were centrifuged at 220 x g for 10 minutes at 4°C, resuspended in 5ml PBS, spun again and finally resuspended in 50 to 1000µl of PBS (depending on cell density). 15µl of cell suspension was dropped onto a clean glass slide, and an equal volume of acridine orange solution (10mg/ml, Sigma) dropped onto a coverslip. The coverslip was carefully lowered onto the slide, and the preparation scored for apoptosis, on the grounds of nuclear morphology, under a fluorescence microscope (Leitz). To determine the minimum number of cells that needed to be scored in order to obtain an accurate estimate of the levels of apoptosis, 550 cells were scored and the results plotted as a running mean (see Figure 5). From this graph, a count of 250 cells was selected as an appropriate estimate and used henceforth.

2.2.5 Preparation of cells for flow cytometry.

Cells were prepared for flow cytometry according to the method of Vindelov *et al.* (1983). Cells were trypsinised, again retaining the medium and PBS wash, spun down (100 x g, 5 min.) and resuspended in PBS at 10^7cells/ml. Aliquots of 100µl were frozen at -70°C prior to analysis. After thawing, 450µl of a cell lysis solution (solution A, see appendix I) was added to the aliquot, mixed gently and incubated at room temperature for 20 minutes. This solution was then neutralised by incubation for 10 minutes at room temperature with 325µl of solution B (see appendix I). Finally, cells were stained by the addition of 250µl of propidium iodide solution (solution C, see appendix I). Samples were then stored on ice and analysed within 24 hours.

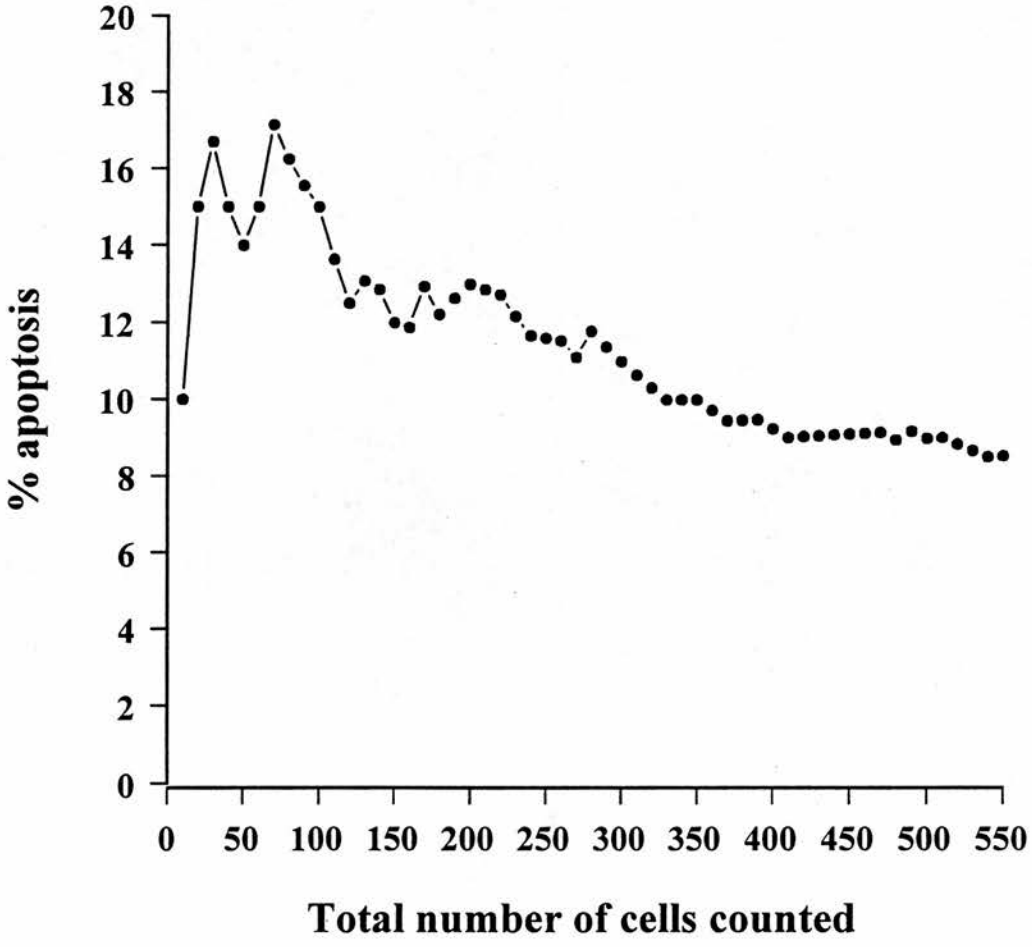


Figure 5: Running mean of percetage apoptosis against cell count.

2.2.6 Flow cytometry.

Analyses were performed on a Coulter EPICS-XL flow cytometer at an excitation wavelength of 488nm. On each occasion of use, the alignment of the laser was checked using Immuno-Check (Coulter) alignment fluorospheres, and half-peak coefficients of variation were less than 2%. Cells were analysed according to forward and side scatter and doublet compensation was performed. At least 10,000 nuclei were analysed in each sample, and the proportion of apoptotic cells estimated as the proportion of cells having a DNA content less than that observed in the G_0/G_1 peak. The cut off point between the G_0/G_1 peak and the hypodiploid peak was defined as the low point closest to the base of the G_0/G_1 peak that showed a count of less than five fluorescent bodies. Bodies having a DNA content less than 12.5% of the G_1 DNA content were assumed to be debris or small apoptotic bodies and were excluded from the analysis. A typical analysis, showing protocols and gating, is shown in Appendix III.

2.3 Chromosomal analyses.

2.3.1 Preparation of metaphase spreads for chromosome counting and FISH analyses.

Cultures were fed 24 hours before harvesting to achieve partial synchronisation. Cultures were harvested when approximately 50% confluent and thus in log phase growth. Two hours before harvesting colcemid (Boehringer Mannheim) was added to the culture medium to a final concentration of 0.1μg/ml. Following the two hour incubation, cells were trypsinised following the usual protocol and centrifuged at 100 x g for 5 minutes. The cells were then gently resuspended in 0.075M KCl and incubated for four minutes at room temperature. Cells were again centrifuged and gently resuspended in a single drop of supernatant before fixing by the addition of 10ml of ice-cold fix (60% methanol, 40% glacial acetic acid). The cell suspension was incubated on ice for 30 minutes, centrifuged and resuspended in 5ml of fresh fix. After a five minute incubation, the cell suspension was spun again and resuspended

in a small volume of fix (depending on the cell density). Slides were prepared by washing in 70% ethanol, rinsing thoroughly in tap water and storing in DDW. Three drops of the cell suspension was dropped onto each slide from a height of approximately 40cm, and the slides air dried. Slides were stored in a dessicator and used within one month.

2.3.2 Chromosome counts.

For chromosome counts, slides were stained in 5% Giemsa prepared in PBS. Thirty metaphase spreads were selected at random and the number of chromosomes counted in each spread.

2.3.3 FISH analyses.

Fluorescent *in-situ* hybridisation (FISH) analyses were performed using a biotinylated murine chromosome 11 paint (Cambio) according to the technique of Fantas *et al.* (1992). Chromosome paints are prepared from PCR amplified DNA obtained from a single chromosome which has been purified using fluorescence activated chromosome sorting, and are thus specific to an entire chromosome rather than a single locus. Slides were warmed at 60°C for 30 minutes and then denatured in 70% formamide (see appendix I) at 65°C for two minutes. Slides were immediately transferred to ice-cold 70% ethanol for two minutes and then through 90% and 100% ethanol (also on ice) for two minutes each. Slides were air dried. After being prewarmed to 42°C and briefly vortexed, the chromosome paint was denatured at 70°C for 8 minutes and pre-annealed at 37°C for 15 minutes. Whilst the paint was pre-annealing, slides and clean coverslips were prewarmed to 37°C. 15µl of the paint was pipetted onto each coverslip, and the coverslips lowered onto slides and sealed with rubber solution. Slides were incubated overnight at 37°C. The rubber seal was removed from the coverslip and the coverslip allowed to fall off in the first wash. Slides were washed four times, for three minutes each in the first wash (2x saline sodium citrate buffer (SSC), 50% formamide) at 45°C. This was repeated with the second wash (2xSSC). Slides were then transferred to 4xSSC, 0.1% Tween 20. Freshly prepared, 40µl aliquots of avidin FITC, biotinylated anti-

avidin and blocking buffer (see appendix I) were spun for 15 minutes at 4°C to remove antibody conjugates. Each slide was incubated for five minutes, at room temperature, with 40µl of blocking buffer. From this point on, care was taken that the slides did not dry out at any stage of the labelling process. The blocking buffer was drained from the slide and replaced with 40µl of avidin FITC, and the slide incubated for 30 minutes at 37°C. Slides were washed three times, for two minutes each in 4xSSC, 0.1% Tween 20, at 45°C. The slide was then incubated for 30 minutes, at 37°C with 40µl of biotinylated anti-avidin. The wash process was repeated, and the slide incubated for a further 30 minutes with 40µl of avidin FITC. Finally, the slides were again washed and mounted with 40µl of vectormount (see appendix I). The slides were examined using the Apple Macintosh-based quantitative image analysis software Macprobe (Perceptive Scientific Instruments Ltd.). This system uses a fluorescence microscope (Leica) equipped with a charged coupled device camera and P-1 filter set (Chroma Technologies). Each fluorochrome is excited separately using a single band-pass excitation filter and grey-level images are captured at x1000 magnification, overlaid and displayed in pseudo-colour. One hundred metaphase spreads were selected at random and the number of stained chromosomes counted in each spread.

2.4 p53 analyses.

2.4.1 Preparation of cytospins.

After trypsinisation, cells were centrifuged at 100 x g for five minutes, the supernatant removed and cells resuspended in PBS at 2 x 10^6/ml. Cytospins were prepared using a Shandon Cytospin 2, Shandon 190005 filter cards and Blue Star slides (Chance Propper Ltd). 100µl of cell suspension was placed in the cytospin chamber and spun onto the slides at 300rpm for 3 minutes. Slides were then air dried and fixed (in 50% methanol, 50% acetone) at 4°C for 5 minutes. Slides were tightly wrapped and stored at -20°C until use.

2.4.2 p53 immunocytochemistry.

The rabbit polyclonal antibody CM5 (Novacastra Laboratories), which is specific for murine p53, was used to detect the induction of p53 subsequent to DNA damage. Slides were placed in Tris buffered saline (TBS, see appendix I) for five minutes. Endogenous peroxidase activity was then blocked by incubating slides for 10 minutes in 3% hydrogen peroxide (Sigma). Slides were rinsed twice in TBS and then washed for five minutes in a third rinse of TBS. Sufficient normal swine serum (NSS, see appendix I) was dropped onto each slide to cover the cells, and slides were incubated for 10 minutes at room temperature. Excess serum was drained from the slide and replaced with the primary antibody (diluted 1:1000 in NSS), and the slides incubated for one hour in a humidified chamber at room temperature. Slides were rinsed and then washed twice, for five minutes each, in TBS. The biotinylated swine-anti-rabbit secondary antibody (Dako) was diluted 1:500 in NSS and incubated on the slides for 30 minutes, in a humidified chamber, at room temperature. Slides were again rinsed and washed twice in TBS. Finally, slides were stained with the Dako ABC HRP kit according to the manufacturers instructions. This kit utilises an avidin and biotin conjugated horseradish peroxidase enzyme complex (ABC). Slides were washed in running water and counter-stained Harris's haematoxylin (Life Science) for seven to 10 seconds. Before mounting in Pertex (Cell Path PLC), slides were dehydrated by two minute incubations in, 64%, 74% and 100% bench alcohol.

2.4.3 Protein sample preparation.

After trypsinisation, cells were centrifuged at 100 x g for five minutes and the supernatant removed. Cells were counted and resuspended in PBS . Samples were snap frozen in aliquots of 10^6 cells and stored at -70°C until use. Upon thawing, samples were washed in PBS, spun down and resuspended in 20µl of lysis buffer (see appendix I). Genomic DNA was sheared by passing the sample through a 23 gauge hypodermic needle or by sonication for 30 minutes, at 37°C. Protein concentrations were calculated using the Bio-Rad protein assay. A 5µl sample of lysate was added to 1ml of the Bio-Rad reagent, incubated at room temperature for five minutes and its

optical density measured at 595 nm. A number of protein standards of known concentration were measured at the same time and a calibration curve plotted. The protein concentration of samples was determined by comparison to the standard calibration curve. Protein samples were kept on ice during processing and stored at -70°C.

2.4.4 Protein electrophoresis and western transfer.

Protein samples were separated on vertical, polyacrylamide gels using the Bio-Rad Mini-Protean II system. One mm thick, 12.5% running gels were prepared (see appendix I) with a 4% stacking gel (see appendix I) and placed in the electrophoresis apparatus with tank buffer (see appendix I). An equal volume of running buffer (see appendix I) was added to 7 µl of protein sample and incubated at 100°C for two minutes. Samples were placed on ice and then loaded on the gel alongside a pre-stained protein molecular weight standard (Gibco). Samples were then electrophoresed at 30mA per gel until the dye front reached the bottom of the gel.

Western transfers were prepared using the Hoefer semi-dry system. The gel apparatus was disassembled and the stacking gel removed. The gel was incubated in transfer buffer (see appendix I) for 10 minutes. Six sheets of Whatman 3MM paper were soaked in transfer buffer and placed on the anode base of the transfer unit. A piece of Highland ECL membrane (Amersham), cut to the size of the gel, was soaked in DDW and placed on top of the 3MM paper. The gel was placed on top of the membrane and three more sheets of 3MM, again soaked in transfer buffer, placed on top. Bubbles were removed by gently rolling with a glass pipette. The gels were transferred in pairs, at 400mA, for 30 minutes. Efficient transfer of proteins was confirmed by staining membranes for 10 minutes in Ponceau S (Sigma). Membranes were rinsed three times in DDW and photographed before de-staining in DDW.

2.4.5 p53 immunohistochemistry.

Non-specific protein binding sites were blocked by incubating the membrane in TBST (tris-buffered saline with Tween, see appendix I) containing 5% w/v

powdered, skimmed, milk for a minimum of one hour. The membrane was then incubated overnight, at 4°C, with the rabbit polyclonal antibody CM5 (Novacastra Laboratories) at a dilution of 1:1000. The membrane was washed three times, for five minutes each, whilst agitating gently, with TBST. The membrane was transferred to a 1:1000 dilution of secondary antibody (horseradish peroxidase conjugated donkey anti-rabbit, Amersham) and incubated for one hour, at room temperature. Finally, the membrane was washed three times, for 10 minutes each, in TBST. Antibody binding was visualised using the ECL detection kit (Amersham) according to the manufacturers instructions. This detection system is based on the oxidation, under alkaline conditions, of luminol by the peroxidase conjugated to the secondary antibody. Decay of the oxidised luminol results in the emission of light which can be detected by autoradiography using blue-light sensitive film (Hyperfilm, Amersham).

2.5 Southern analyses.

2.5.1 Preparation of high molecular weight genomic DNA.

In most instances, high molecular weight genomic DNA was prepared from cell pellets containing approximately 1 x 10^6 cells. Cells were resuspended in 500µl of lysis buffer (see appendix I) and 30µl of proteinase K solution (20mg/ml) added. The lysates were then shaken in an orbital shaker, at 220rpm, at 37°C, overnight. An equal volume of phenol was added to the lysate, the tube inverted several times and briefly spun down. The upper, aqueous phase was carefully removed leaving behind any precipitates formed at the boundary between the two phases. The phenol extraction was repeated and two chloroform (see appendix I) extractions performed in a similar way. The DNA was precipitated from the resulting solution with an equal volume of propan-2-ol, lifted out of the tube with a sterile pipette tip and placed in a fresh tube containing 70% ethanol. The DNA was then centrifuged at 13,000rpm, in a bench-top microfuge, for 15 minutes. The ethanol was drained from

the tube, and the DNA air dried for 30 minutes. Finally, the DNA was dissolved in 60µl of tris/ethylenediaminetetraacetic acid (EDTA) buffer (TE, see appendix I). Occasionally, DNA was prepared from solid tissue. The tissue (approximately 100mg) was finely chopped, with a scalpel, in a petri dish containing 5mls of sodium/tris/EDTA buffer (TNE, see appendix I). The resulting cell suspension was centrifuged at 1000 x g, for 15 minutes and resuspended in 10mls of TNE. Sodium dodecyl sulphate (SDS) was added to a final concentration of 0.5% (W/V), and proteinase K to a concentration of 50µg/ml. The cell suspension was then incubated at 50°C for one hour. One phenol extraction was performed and the aqueous phase removed with a wide bore pipette. A second extraction was similarly performed with chloroform. DNA was precipitated by the addition of two volumes of pure ethanol and spooled out with a sealed pasteur pipette. The pellet was washed with 70% and then 100% ethanol, and air dried for 2-3 hours. The DNA was dissolved in TE and the concentration and purity determined spectrophotometrically.

2.5.2 Restriction endonuclease digestion and agarose gel electrophoresis of high molecular weight genomic DNA.

Genomic DNA samples (10-15µg) were digested with appropriate restriction endonuclease enzymes using the incubation buffer and reaction conditions supplied by the manufacturer (Life Technologies). Spermidine was added to a final concentration of 3mM, and 2-10 units of enzyme used per µg of DNA digested. Digestions were performed at 37°C, overnight, and then stored at 4°C until electrophoresed. After the addition of tracking dye (see appendix I), samples were electrophoresed alongside DNA molecular weight markers (λ HindIII fragments, Life Technologies) in 0.6-0.8% horizontal, submarine, agarose gels (see appendix I). Gels were run at 4°C, for 16 hours, at 65V.

2.5.3 Southern transfer of DNA fragments to nylon membranes.

After electrophoresis, gels were stained for 30 minutes in 0.5µg/ml ethidium bromide and photographed under UV light. The DNA marker lanes were trimmed from the gel and the gel depurinated for 15 minutes in 0.25M HCl. The gel was then

denatured in alkali buffer (see appendix I) for one hour, at room temperature, with gentle agitation. The alkali buffer was neutralised by gently agitating the gel in neutralising buffer (see appendix I) for one hour. The gel was then transferred to 10xSSC (see appendix I) while the transfer was constructed. Half a box of Kleenex tissues were placed in the bottom of a plastic tray and covered with several layers of Grade I Whatman filter paper. Two sheets of filter paper soaked in 10xSSC were placed on top of the dry sheets and the nylon membrane (Hybond-N, Amersham), also soaked in 10xSSC, placed on top of these, taking care to exclude bubbles. The gel was then placed on top of the membrane, again excluding bubbles, and covered in another sheet of pre-soaked filter paper which had been cut to the size of the gel. A synthetic sponge was immersed in 10xSSC and allowed to drain until drips no longer fell from the sponge when held horizontal. This was placed on top of the last sheet of filter paper, and the entire apparatus sealed in a polythene bag and left overnight. The apparatus was then dismantled and the membrane rinsed in 3xSSC. DNA was bound to the membrane by baking at 80°C for two hours.

2.5.4 DNA probes.

The recombinant plasmid pCPR4.1 (Purdie, 1993), which contains both 5' and 3' regions of the *p53* gene sequence, was digested with HindII and the 2kb fragment (containing *p53* exons 10 and 11) purified (see section 2.5.5) and used as a DNA probe. The *neo* probe was a 600bp, PstI fragment derived from the recombinant plasmid pMC1-Neo (Thomas and Capecchi, 1987). The *Hprt* polymerase chain reaction (PCR) product derived from complimentary DNA (cDNA) of the ES cell line E14 (see sections 2.6.1 to 2.6.3) was used as a probe for *Hprt*.

2.5.5 DEAE membrane purification of DNA fragments for DNA probes.

DNA fragments were purified for use as probes using DEAE (Schleicher and Schuell) membrane according to Sambrook *et al.* (1989). Briefly, the DNA was digested with the appropriate restriction enzyme and electrophoresed in an agarose gel (see section 2.5.2). A slit was cut in the gel, just in front of the required band, and a piece of DEAE membrane placed in the slit. The band was electrophoresed

onto the membrane and then eluted from it by incubating at 65°C, for one hour, in a high salt buffer (50mM Tris, 1mM EDTA, 1M NaCl, pH 9.0). The resulting DNA solution was extracted once with phenol:chloroform and then precipitated with ethanol (see section 2.5.1). The DNA was centrifuged at 13,000rpm, in a bench-top microfuge, for 15 minutes. The ethanol was drained from the tube and the pellet washed in 70% ethanol. The DNA was again centrifuged for 15 minutes at 13,000rpm, the ethanol removed and the DNA air dried for 30 minutes. Finally, the DNA was dissolved in TE and quantitated spectrophotometrically.

2.5.6 Labelling of DNA probes.

DNA probes were labelled with α^{32}P deoxycytosine triphosphate (dCTP) using the random priming method. The Prime-It RmT kit (Stratagene) was utilised for this purpose and used according to the manufacturers recommended protocol. In summary, 50ng of probe was added to a single-use reaction tube containing random primers, nucleotides, buffer and co-factors for use with α^{32}PdCTP and denatured by boiling for 5 minutes. To this tube was added 50μCi of α^{32}PdCTP (ICN) and 12 units of DNA polymerase, and the contents incubated at 37°C for 30 minutes. After this time the reaction was terminated by the addition of 2μl stop mix (0.5M EDTA, pH8.0). Unincorporated α^{32}PdCTP was removed with a Sephadex G-50 nick column (Pharmacia), used according to the manufacturers instructions. The column was rinsed with TE and equilibrated by allowing 3ml of TE to pass through the column. The labelled DNA probe was added, along with 400μl TE, and allowed to pass into the column bed. The probe was then eluted from the column by the addition of a further 400μl of TE and collected in an eppendorf. An approximate measure of isotope incorporation into the probe was obtained by comparing the Geiger counter reading of the tube relative to the column.

2.5.7 Hybridisation of labelled DNA to Southern transfers and post-hybridisation treatment.

Southern transfers were placed, DNA side up, on a sheet of fine nylon mesh, rolled and placed in a Hybaid hybridisation bottle. The bottle was filled with 3xSSC and

tapped to remove bubbles. An aliquot of sonicated salmon sperm DNA (Sigma) was denatured by boiling for five minutes and added to hybridisation buffer (see appendix I) at a concentration of 100μg/ml. The 3xSSC was removed, and replaced with 15mls of hybridisation buffer. The hybridisation bottle was placed in a Hybaid Mini 10 hybridisation oven, and prehybridised for four hours at 65°C. The α^{32}PdCTP labelled DNA probe (see section 2.5.6) was denatured, by boiling for five minutes, and added to the hybridisation bottle. The bottle was then incubated overnight, at 65°C.

Post-hybridisation wash solutions were prewarmed to 65°C. The hybridisation buffer was removed from the bottle and the bottle rinsed with 200ml of wash 1 (see appendix I). A second aliquot of 200ml of wash 1 was added to the bottle and incubated for 10 minutes at 65°C, in the Hybaid oven. This wash was repeated once. 200ml of wash 2 (see appendix I) was added to the bottle, and the bottle incubated for two hours at 65°C. This wash was repeated, and then the bottle rinsed twice with 2xSSC. The filter was removed from the bottle and sealed in cling-film. The sealed filter was placed in an X-ray cassette (Hypercassette, Amersham) containing two Hyperscreen (Amersham) intensifying screens and a sheet of Kodak X-OMAT film, and exposed at -70°C for 1-10 days. The exposed film was developed in an Hyperprocessor (Amersham International plc) automatic film developer. After autoradiography, the filter was stripped of hybridised probe by placing in a boiling solution of 0.1% SDS, in which the filter was left to cool to room temperature.

2.6 *Hprt* mutation spectrum analysis.

2.6.1 RNA preparation.

In order to avoid contamination with RNAase, pre-packaged filter tips and sterile, disposable plastic-ware were used. RNase-free DDW, prepared using the Elga Maxima system (Elga), was used. Gloves were worn at all times and care was taken not to touch any surfaces that would come into contact with the RNA preparation. The phenol based Trizol reagent (Life Technologies) was used for RNA extractions.

Log phase cells (approximately 3 x 10^6) were lysed, in their culture dish, by the addition of 2ml of Trizol. The lysate was split into two Eppendorfs and incubated for five minutes at room temperature before the addition of 200µl of chloroform and 300µl of DDW. The homogenised samples were vortexed and then incubated for a further three minutes. The samples were centrifuged at 13,000rpm in a bench-top microfuge for five minutes and the upper aqueous phase removed. If the aqueous phase was slightly cloudy, an extra chloroform extraction was performed using an equal volume of chloroform. RNA was precipitated from the sample by the addition of 0.5ml propan-2-ol and incubation at -70°C, for 1 hour. The RNA samples were centrifuged, at 13,000rpm, in a microfuge for 10 minutes at 4°C, washed in 200µl of 70% ethanol and air dried for 30 minutes. The RNA was dissolved in 100µl of DDW, and stored, in aliquots, at -70°C. The concentration and purity of RNA samples was determined spectrophotometrically.

2.6.2 cDNA preparation.

A 2µg aliquot of RNA was defrosted on ice and 0.5ng of oligo(dT) primers (Life Technologies) added. The mixture was heated to 72°C for 10 minutes and transferred to ice for 1 minute. To the tube were added 2µl PCR buffer (Life Technologies), 2µl of 10µM mixed dNTPs (deoxynucleoside triphosphates, Life Technologies), 2µl 0.1M dithiothreitol (DTT, Life Technologies), 1µl (200U) of M-MLV reverse transcriptase (Life Technologies) and sterile DDW to a final volume of 20µl. The tube was incubated at 37°C for one hour and the reaction terminated by heating to 80°C for 15 minutes. Samples were stored at 4°C.

2.6.3 The polymerase chain reaction.

Two PCRs were performed on each cDNA sample using the same PCR conditions and reagents. The first reaction was designed to amplify an 832 bp fragment encompassing the entire coding sequence of the *Hprt* gene. As the samples analysed were derived from cell lines assumed to be bearing γ-irradiation induced *Hprt* mutations (see section 2.1.7), it was predicted that a proportion of the samples would have large deletions in or around the region of the *Hprt* gene, and thus might not

produce a PCR product. The second reaction was therefore designed as a control to ensure that the absence of an *Hprt* PCR product was due to the deletion of a primer site and not a poor cDNA preparation or PCR. The control reaction was designed to amplify a 1265 bp region of the *Tbp1* gene, a constitutively expressed ATPase. All PCR reactions were repeated at least once. Sequences (5' to 3') of PCR primers are given in Table 1.

Table 1: Names and sequences of PCR primers.

Primer name	Sequence
HPRT1	ctcactgctttccggagc
HPRT2	ccacaagatcaggacaccgg
TBP1up	ttccatctgtccacggaagagattgtc
TBP1rp	ctggtcgactagaggtgtcccctaggc

Reactions were carried out in 0.5ml eppendorf tubes on an Omnigene thermocycler (Hybaid). Each reaction contained 200μM of each dNTP (Pharmacia), 1μM of each primer (Oswel), 1.5mM $MgCl_2$ (Life Technologies), 1 unit *Taq* DNA polymerase (Life Technologies), 1x PCR buffer (Life Technologies) and 2μl of cDNA template (see section 2.6.1). The reaction volume was made up to 50μl with DDW and 50μl of paraffin oil was overlaid to prevent evaporation. Reactions were denatured at 94°C for three minutes and then 30 cycles of amplification were carried out with each cycle consisting of 45 seconds of denaturation at 95°C, 30 seconds annealing at 60°C and one minute, 30 seconds extension at 72°C. Reactions were visualised on a 1% agarose gel (see section 2.5.2).

2.6.4 Sequencing of PCR products.

Direct sequencing of PCR products was carried out using the Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham) according to the

manufacturers instructions. This method of cycle sequencing utilises [α-^{33}P]dideoxynucleotide (ddNTP) terminators in conjunction with a thermostable DNA polymerase. Because only properly terminated DNA chains are labelled, "stop" artefacts and most background bands are eliminated.

Prior to sequencing, excess dNTPs and PCR primers were removed by incubating 5μl of PCR product with 10 units of exonuclease I (Amersham) and two units of shrimp alkaline phosphotase (Amersham), at 37°C for 15 minutes. The reaction was terminated by incubation at 80°C for 15 minutes. An aliquot of untreated PCR product was quantitated by agarose gel electrophoresis and comparison against a quantitative marker.

A 2μl aliquot of dGTP termination master mix was mixed with 0.5μl of [α-^{33}P]ddNTP to produce a termination mix for each ddNTP. A reaction mixture was made for each sample by mixing 25fmol (usually 1-5μl) of PCR product with 1pmol sequence-specific primer (either HPRT1 or HPRT2) and 2μl reaction buffer, in a final volume of 20μl. 4.5μl of reaction mixture was transferred to each termination tube, mixed and overlaid with one drop of paraffin oil. The tubes were placed on a thermal cycling block and amplified under the same conditions as the original PCR reaction (see section 2.6.3). Stop solution (4μl) was added to each tube and the samples heated to 70°C for 2 minutes before loading 3μl per lane onto a denaturing polyacrylamide gel.

2.6.5 Sequencing gel electrophoresis.

Sequencing was carried out on BRL S2 (Life Technologies) sequencing apparatus using 0.4mm spacers and sharks-tooth combs. Plates were washed thoroughly and wiped with 70% ethanol before use. Immediately before pouring, 60ml of gel stock solution (see appendix I) was polymerised by the addition of 60μl 25% ammonium persulphate (Life Technologies) and 60μl TEMED (Fisher). The gel was allowed to polymerise, horizontally, for 1 hour with inverted combs. The gel was then pre-run in glycerol tolerant gel buffer (USB), without combs, for 30-60 minutes at 90W, until it reached 50°C. The combs were replaced to form wells, which were rinsed

thoroughly with running buffer. Samples were heated to 75°C for 3 min prior to loading and run for 2-6 hours at 80W. The gel was fixed twice in 10% acetic acid 10% methanol for 5 minutes and transferred onto 3MM paper. Finally, the gel was dried for 2 hours on a vacuum gel dryer (Bio-Rad) and exposed to Kodak Biomax-MRI film overnight.

2.7 Statistical methods.

All statistical analyses were performed using the statistical computer package Minitab Release 11 (Minitab). Datasets were tested for normal distribution and homogeneity of variance and where these criteria were not met, non-parametric tests were utilised.

CHAPTER THREE

RESULTS

Section one:
The immediate response to DNA damage in ES cells.

3.1.1 The apoptotic response to DNA damage in ES cells.

In order to determine the response of ES cells to a variety of stimuli, ES cells were exposed to a number of DNA-damaging agents. These included the topoisomerase II inhibitor etoposide, the topoisomerase I inhibitor camptothecin, UVC-irradiation and γ-irradiation (see chapter 2, section 2.2 for a discussion of the types of DNA-damage induced by these agents). In the first instance, cells were stained with acridine orange and apoptotic cells recognised by their condensed and often fragmented chromatin. This method of detection allows a definitive assessment of the proportion of apoptotic cells based on their nuclear morphology but, although widely accepted, it is time consuming. Therefore, a flow cytometric method based on the proportion of cells having a DNA content less than that observed in the G_0/G_1 peak (the hypodiploid peak) was also used. The accuracy of this estimate was confirmed by comparison to direct measurements based on nuclear morphology. In this comparison, cells were irradiated with $17J/m^2$ UVC and the irradiated cells divided into two aliquots, one to be analysed by morphometric criteria following acridine orange staining and the other by flow cytometry (Figure 6). A statistical analysis showed a high correlation between these two methods (Pearson correlation coefficient = 0.929). A similarly high correlation was seen between flow cytometric and morphometric data obtained after γ-irradiation.

3.1.1.1 The apoptotic response to Camptothecin.

Wild-type (CGR8) ES cells were exposed to Camptothecin levels ranging from 0.01 to 1μM and cells harvested for acridine orange counts at 0, 12, 24, 36 and 48 hours.

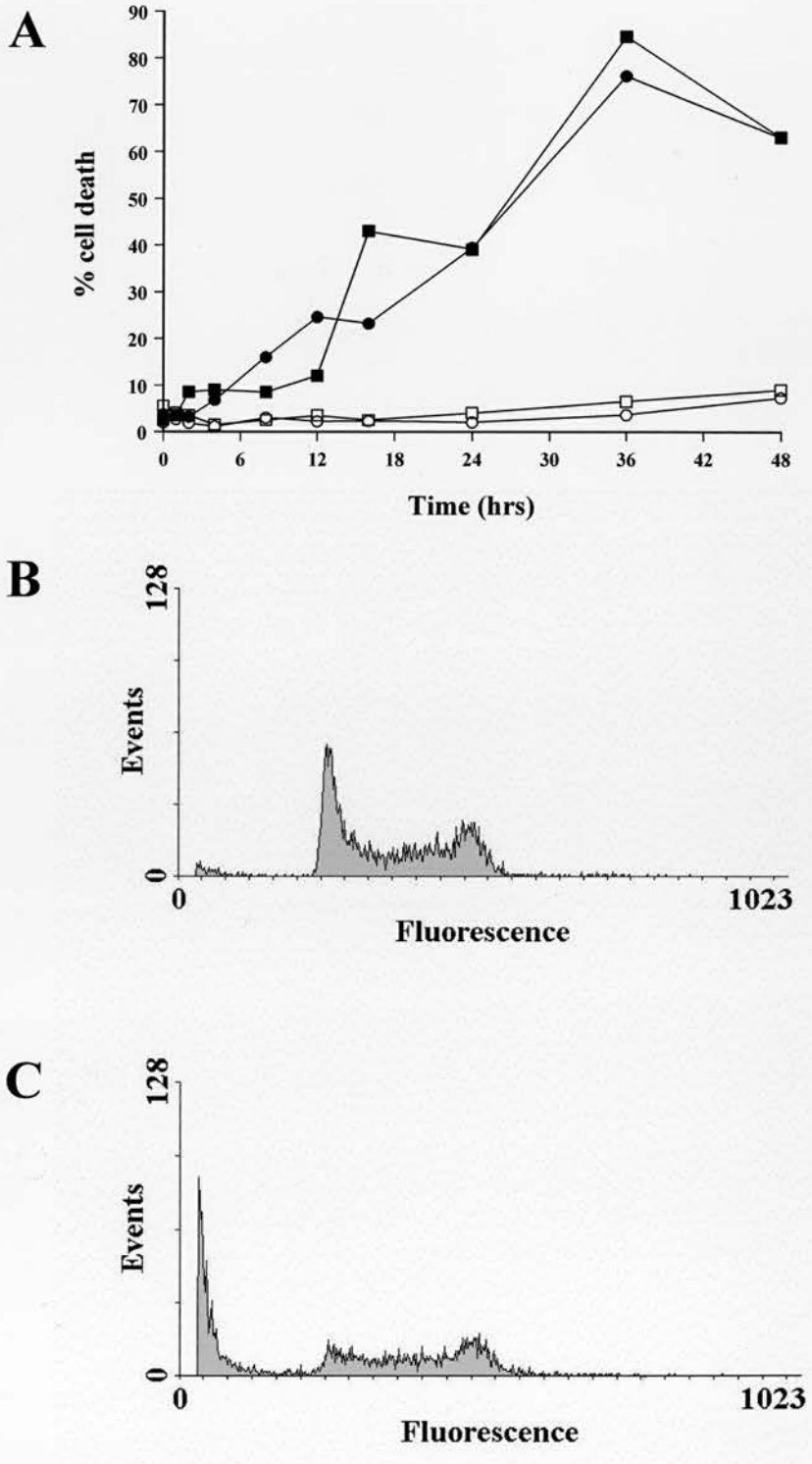


Figure 6: A: Kinetics of the induction of apoptosis in response to 17J/m^2 UVC. Solid symbols reprersent treated ES cells (E14) and open symbols mock-treated controls. Circles represent estimates obtained by flow cytometry and squares the same cells stained with acridine orange and counted directly. B: DNA histogram of mock-treated ES cells 24hrs subsequent to treatment. C: DNA histogram of ES cells treated with 17J/m^2 UVC 24hrs after treatment.

All treatments were performed in duplicate. Only the highest dose used (1μM) induced apoptosis to any significant degree (Figure 7). Elevated levels were first observed at 36 hours and continued to rise to a maximum of 85% at 48 hours.

3.1.1.2 The apoptotic response to Etoposide.

ES cells (CGR8) were exposed to a variety of concentrations of etoposide ranging from 0.2 to 20μM and cells harvested for acridine orange counts at 0, 12, 36 and 48 hours. All treatments were performed in triplicate. Very little apoptosis was seen in the DMSO controls and the lowest dose (0.2μM) of etoposide used. In the two higher doses (2μM and 20μM), substantial numbers of apoptotic cells were seen at the 36 hour time-point, and this proportion had increased still further by 48 hours (Figure 8).

3.1.1.3 The apoptotic response to UVC-irradiation.

E14 cells were exposed to UVC-irradiation (in triplicate) over a dose range of 23 to 1100J/m^2. Cultures were observed by phase contrast microscopy after 20 hours and appreciable levels of cell death were visible in all but untreated controls. Cultures were therefore harvested for acridine orange counts at 24 hours. A log linear relationship was observed between UVC dose and apoptosis (Pearson correlation coefficient between percentage apoptosis and $\log^{10}$(dose) = 0.903; Figure 9).

A UVC dose of 93J/m^2, which induced 55% apoptosis at 24 hours, was used to investigate the kinetics of the induction of apoptosis. Cell cultures, again in triplicate, were harvested at time-points ranging from 0 to 40 hours. Elevated levels of apoptosis were seen as little as four hours after irradiation and continued to rise, reaching a plateau at 24 hours (Figure 10). Similar levels of apoptosis were observed with a UVC dose of 17J/m^2 (Figure 6) but at this dose the kinetics of induction were slightly slower, peaking at 36 hours.

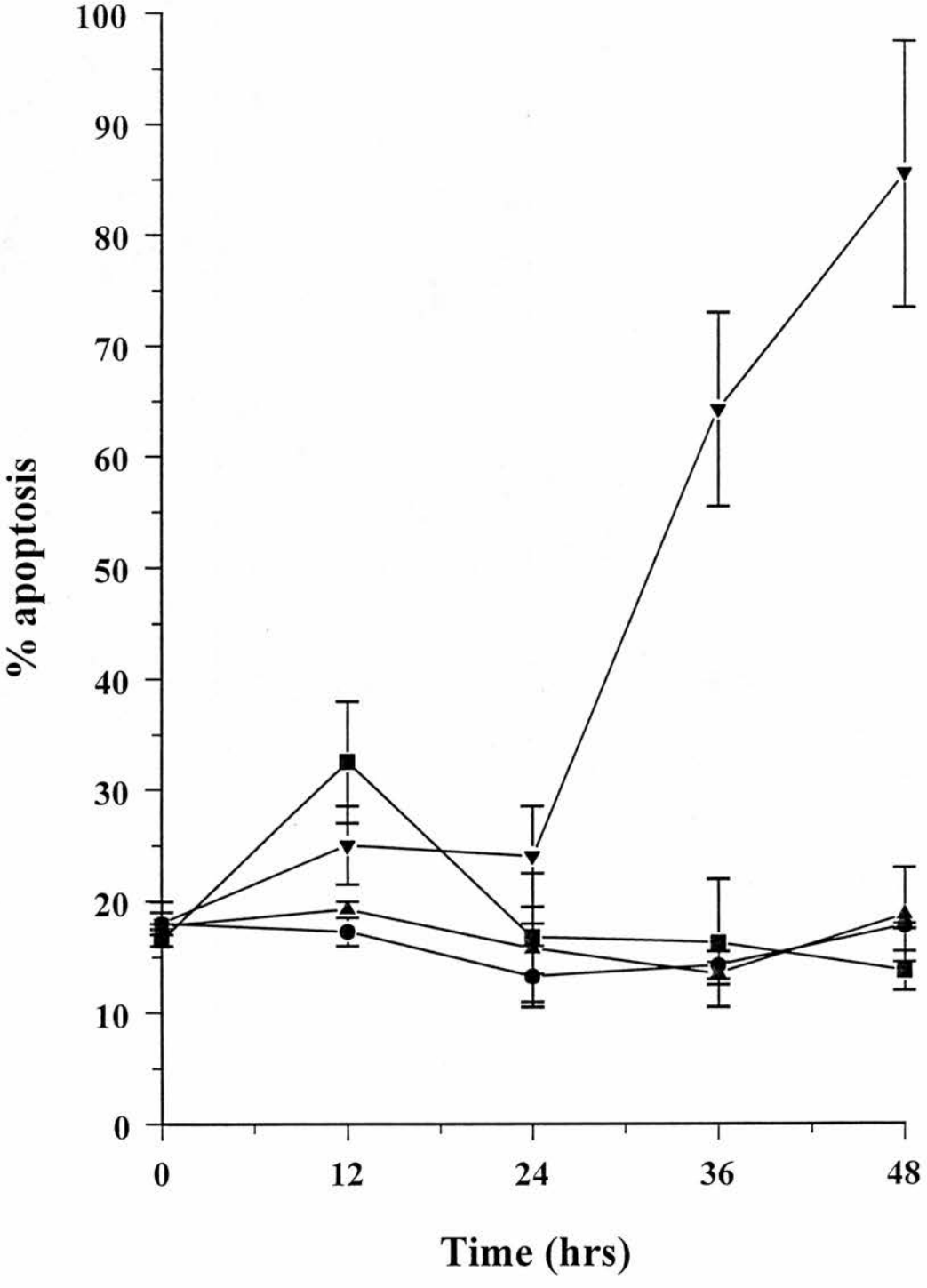


Figure 7: Kinetics of the induction of apoptosis in wild-type ES cells (E14) in response to a range of doses of camptothecin. Circles, 0.2% DMSO control; squares, 0.01μM camptothecin; upward pointing triangles, 0.1μM camptothecin; downward pointing triangles, 1μM camptothecin. Each data point represents the mean of two duplicates and error bars indicate the range.

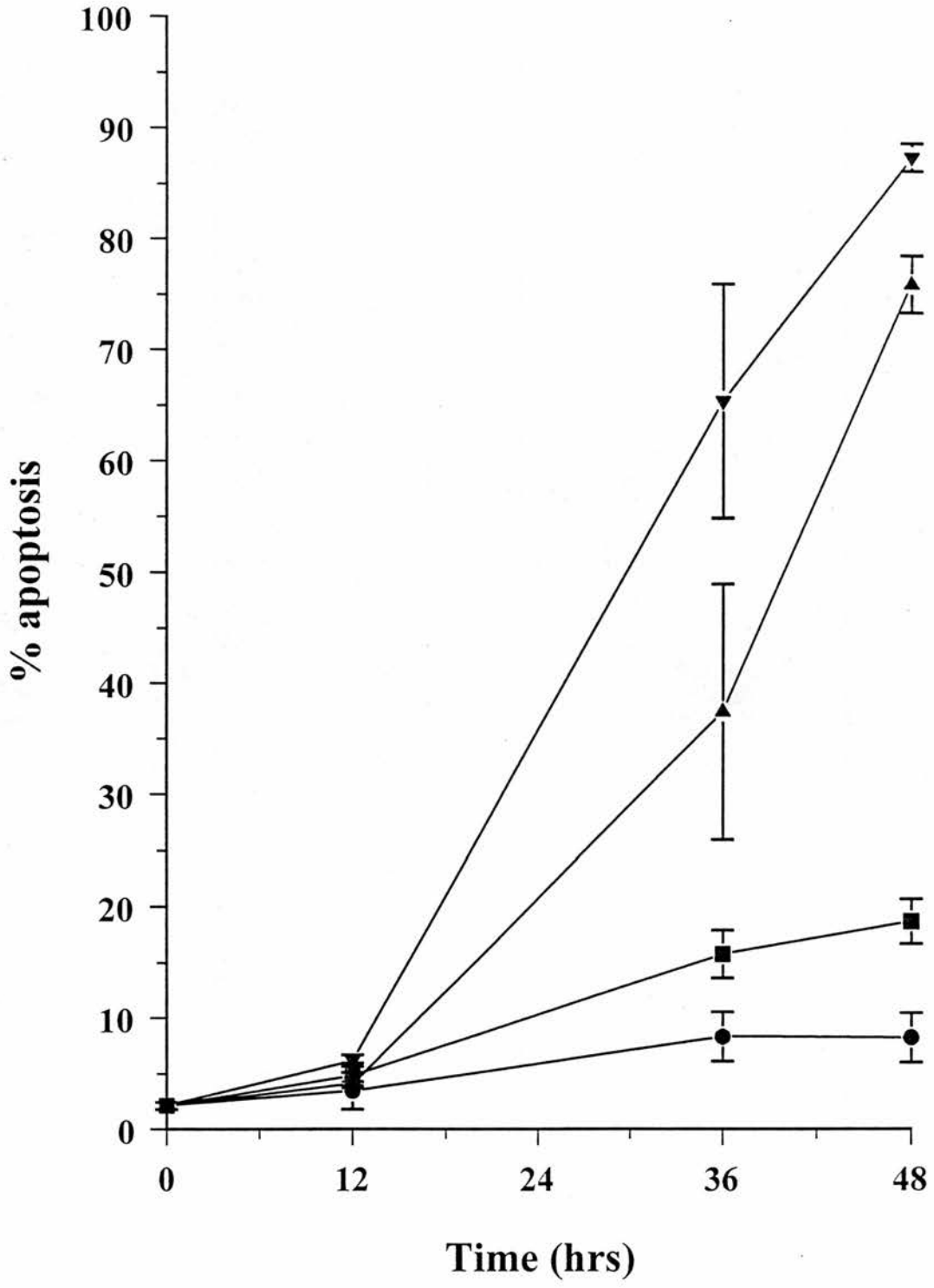


Figure 8: Kinetics of the induction of apoptosis, in wild-type ES cells (E14), in response to a range of doses of etoposide. Circles, 0.2% DMSO control; squares, 0.2μM etoposide; upward pointing triangles, 2μM etoposide; downward pointing triangles, 20μM etoposide. Each data point represents the mean of three replicates and error bars indicate the standard error.

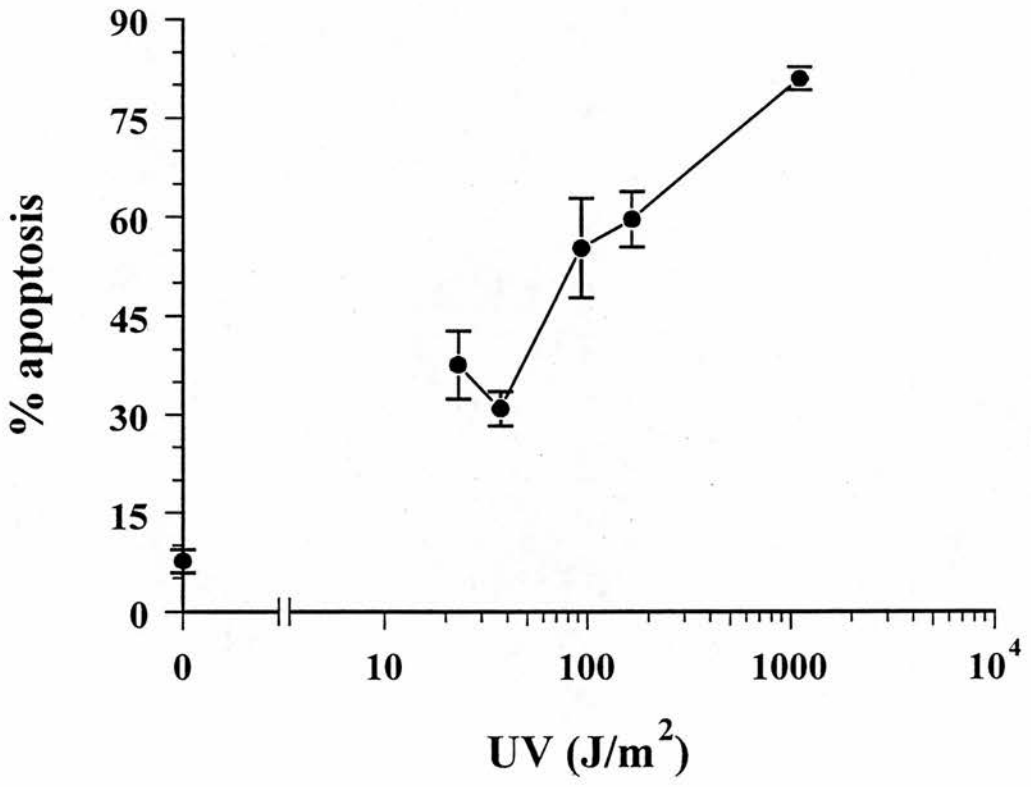


Figure 9: Dose response of apoptosis 24 hours after UVC-irradiation in wild-type ES cells (E14). Each data point represents the mean of three replicates and error bars indicate the standard error.

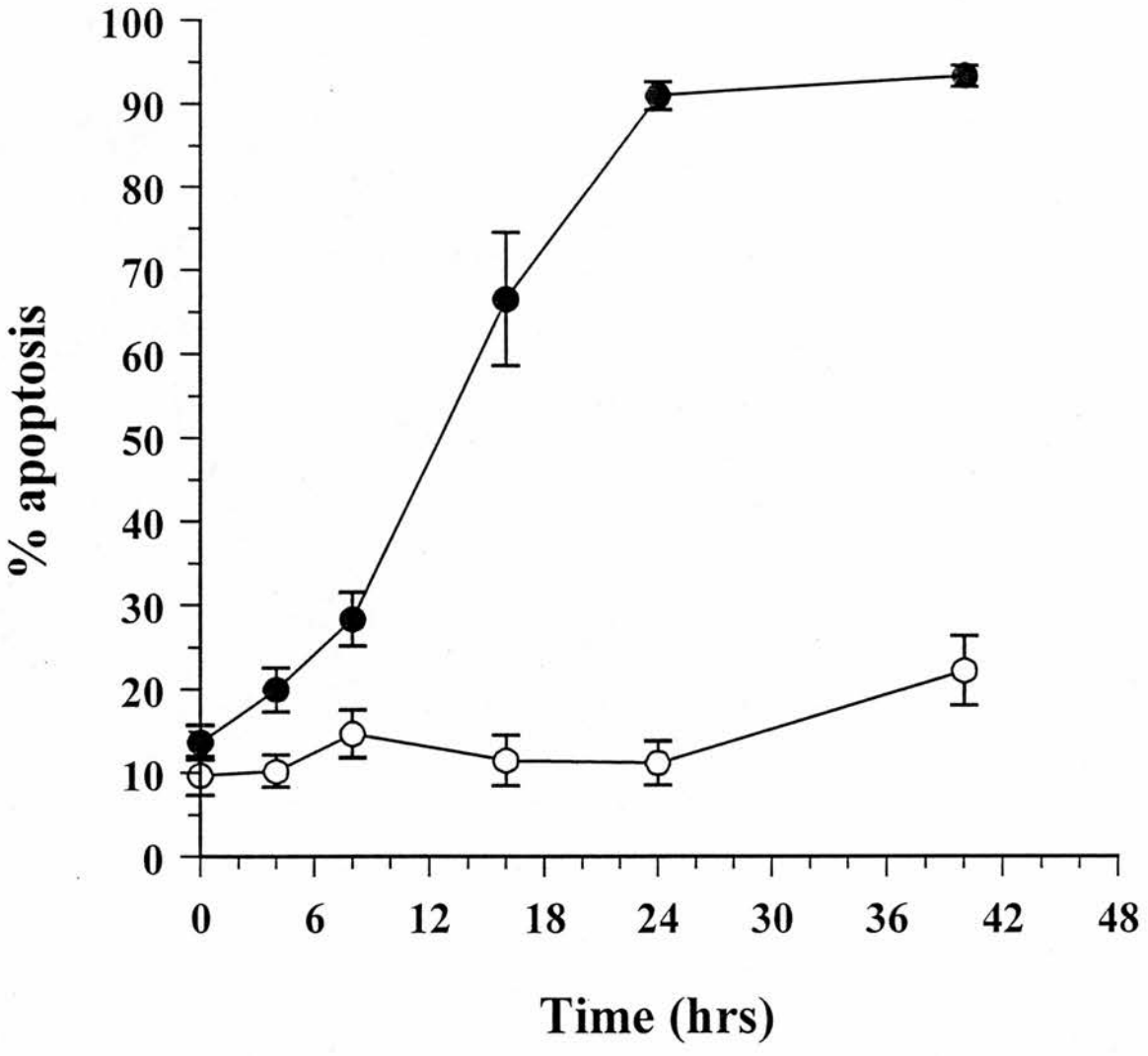


Figure 10: Kinetics of the induction of apoptosis in wild-type ES cells (E14) subsequent to 93J/m^2 UVC-irradiation. Open symbols, mock-treated controls; closed symbols, irradiated cells. Each datapoint represents the mean of three replicates and error bars indicate standard errors.

3.1.1.4 The apoptotic response to γ-irradiation.

Duplicate cultures of E14 cells were exposed to γ-radiation doses ranging from 3.6 to 19Gy and harvested after 0, 24 48 and 72 hours. The level of apoptosis was assessed by acridine orange staining. Even at the highest dose of 19Gy, levels of apoptosis were low (less than 40%) relative to those observed after both UVC-irradiation and topoisomerase inhibitors (Figure 11). In addition, the onset of the induction of apoptosis appeared to be delayed and levels were still rising at 72 hours. Because of the effect of cellular proliferation on the denominator, it was not possible to extend the time-course beyond 72 hours using this method of detection. Two further experiments, using flow cytometry, to examine the kinetics of apoptosis after γ-irradiation (7.6Gy) revealed little or no induction of apoptosis (Figures 12 and 13). These findings were unexpected and led to concerns over the degree of variation in levels of apoptosis between the experiments. To address this issue, three more time-courses were performed (in duplicate) and analysed by flow cytometry. A strong induction of apoptosis was not seen in any of the experiments but variation between experiments was relatively high (Figure 14). In all three experiments, levels of apoptosis were consistently slightly higher in irradiated cells but a statistical analysis of the entire data-set showed that this difference was not significant (Analysis of Variance (ANOVA), $p=0.092$).

3.1.1.5 The effect of varying levels of confluence on the apoptotic response to UVC-irradiation

Levels of apoptosis induced after DNA-damage were often quite variable between experiments (see sections 3.1.1.4 and 3.2.2.2). In an attempt to determine the source of this variation, the effect on the induction of apoptosis of variations in confluence levels of cells immediately prior to treatment was examined. In each of the previous experiments, the confluence level of cells to be treated was not accurately controlled between experiments. To investigate the effect of this parameter on apoptosis, cell cultures were seeded at varying densities, irradiated with $17J/m^2$ UVC and harvested for flow cytometry 24 hours later. Cell counts were obtained, at the time of irradiation, from control cultures seeded at the same density as cultures to be treated.

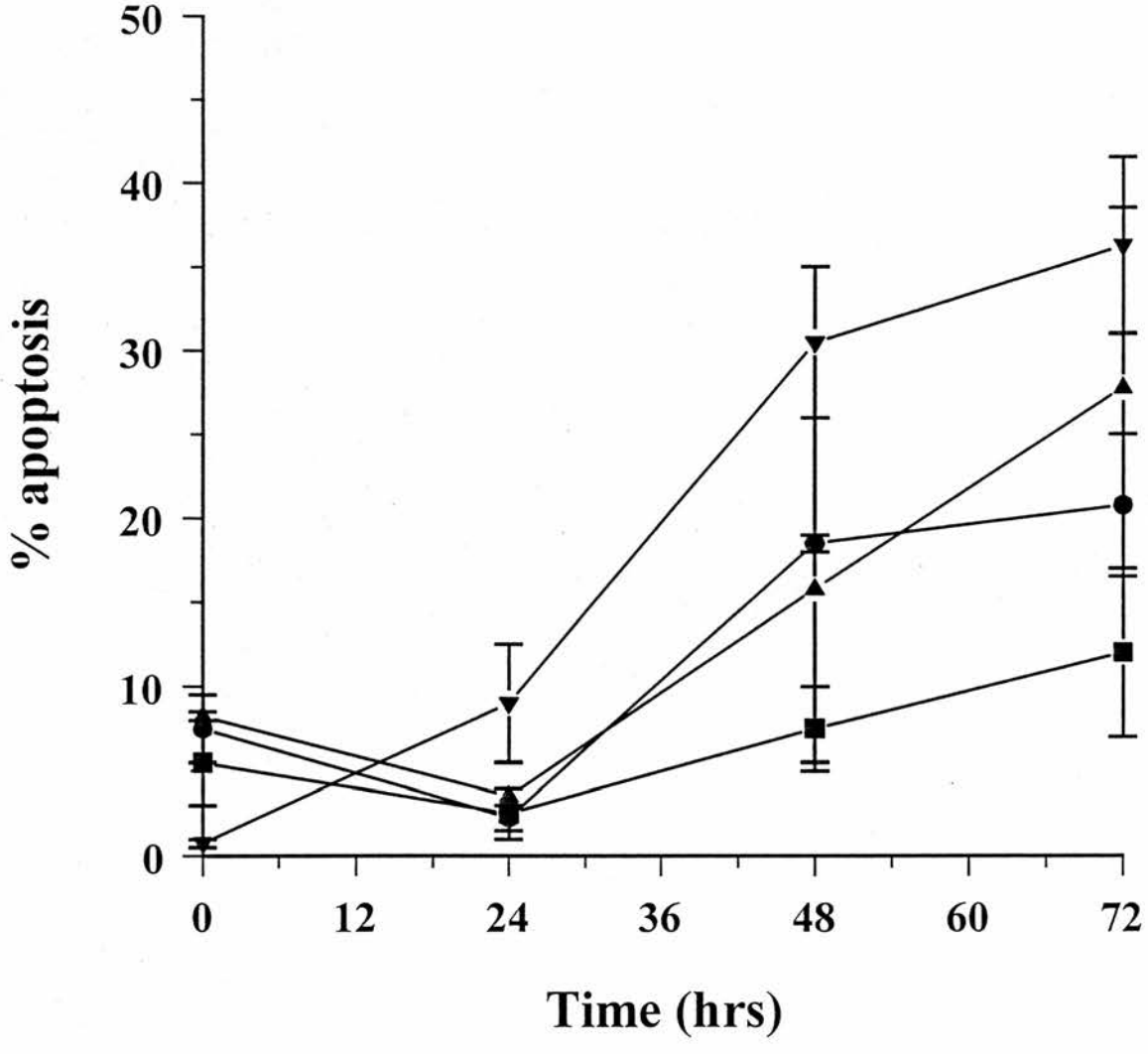


Figure 11: The kinetics of the induction of apoptosis in wild-type ES cells (E14) in response to a range of doses of γ-irradiation. Squares, unirradiated control; circles, 3.8Gy; upward pointing triangles, 7.6Gy; downward pointing triangles, 19Gy. Each data point represents the mean of two replicates and error bars indicate the range.

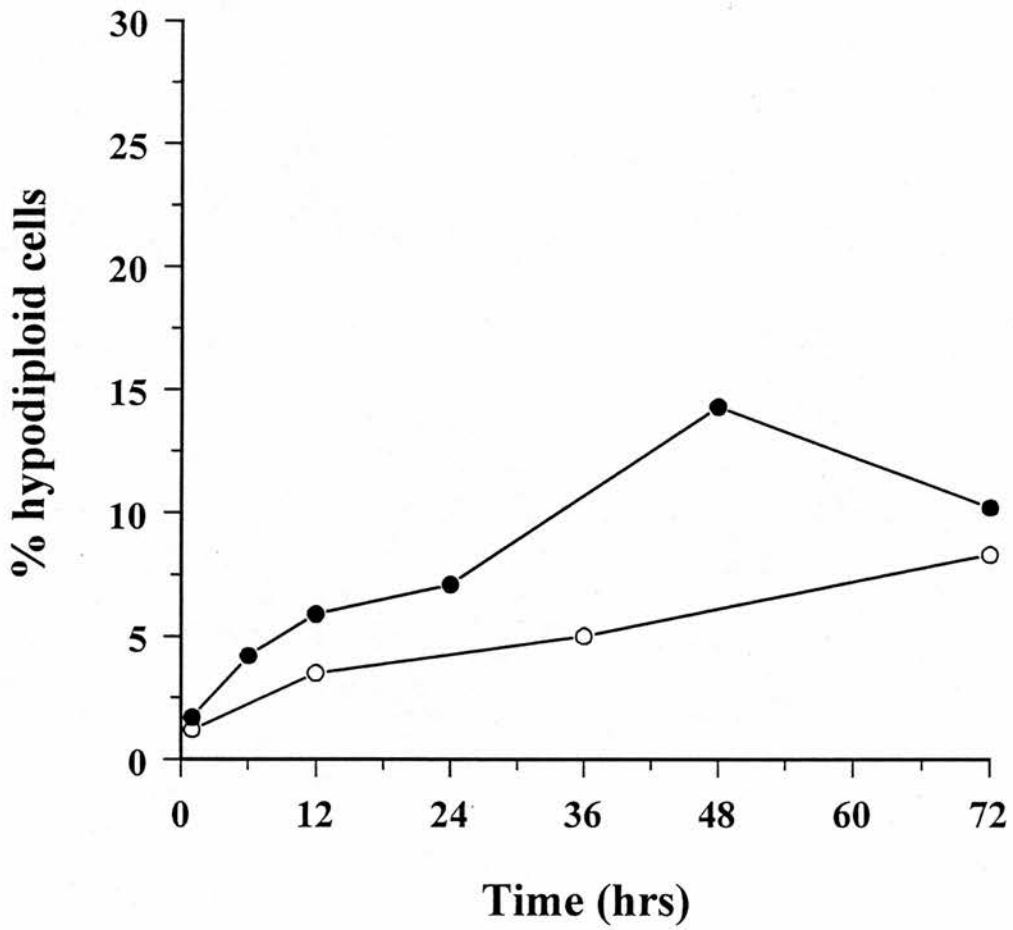


Figure 12: Kinetics of the induction of apoptosis in wild-type ES cells (E14) following γ-irradiation. Open circles, unirradiated cells; closed circles, irradiated cells (7.6Gy).

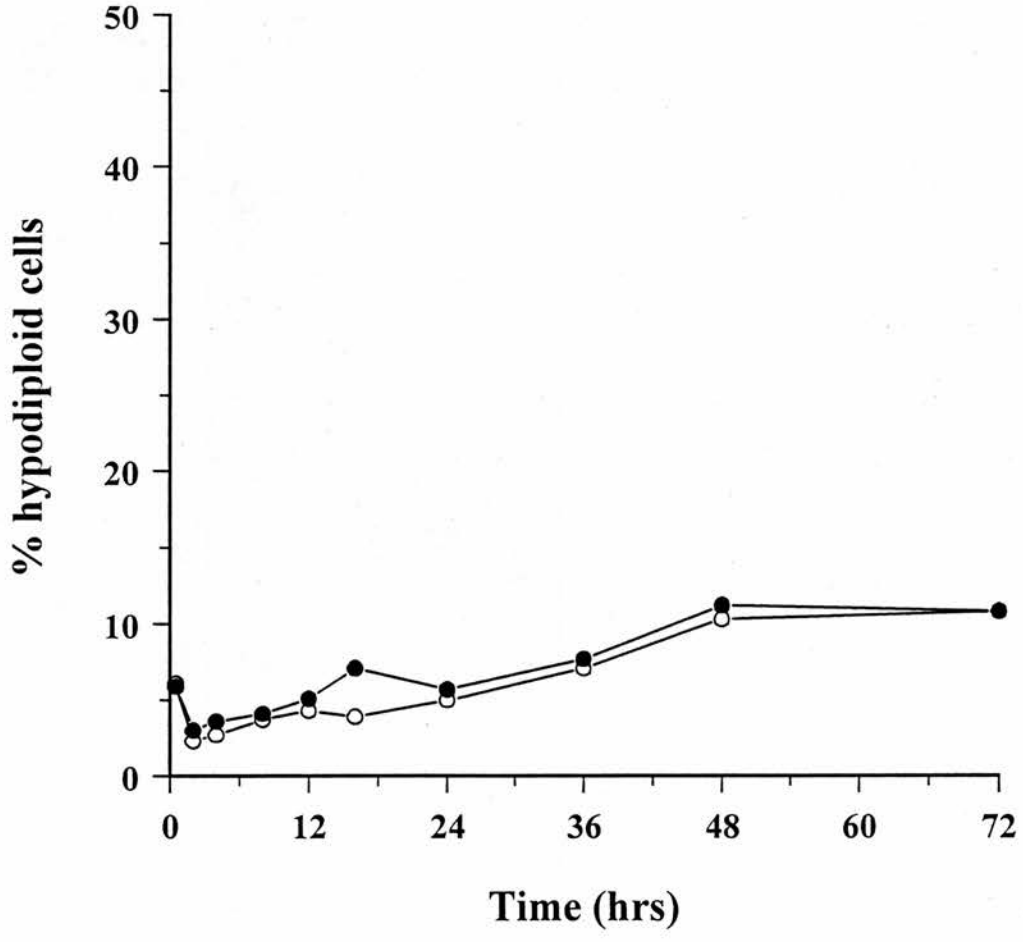


Figure 13: Kinetics of the induction of apoptosis in wild-type ES cells (E14) following 7.6Gy γ-irradiation. Open symbols, mock-treated controls; closed symbols, irradiated cells.

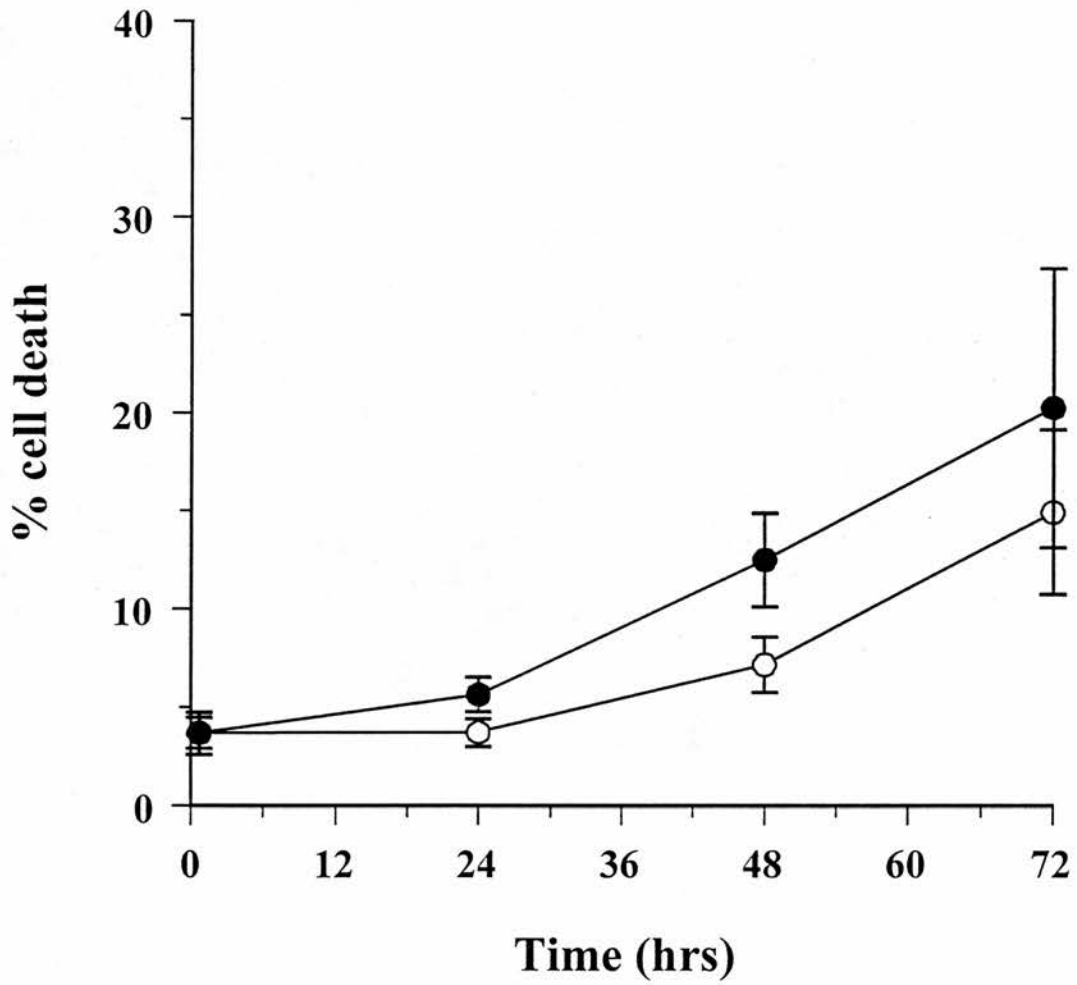


Figure 14: Kinetics of the induction of apoptosis in wild-type ES cells (E14) following 7.6Gy γ-irradiation. Open symbols, mock-treated controls; closed symbols, irradiated cells. Each datapoint represents the mean of three experiments and error bars indicate the standard error.

In total, four experiments were performed. Although it was not possible to statistically test this hypothesis, all four experiments suggested a possible trend whereby cells treated at either very low or very high levels of confluence were more resistant to the induction of apoptosis (Figure 15). However, there was appreciable variation in the response between experiments indicating that confluence alone could not account for the disparity in levels of induction of apoptosis. It should also be stressed that these experiments examined the effect of confluence on levels of apoptosis at a single time-point and thus the possibility that confluence does not affect sensitivity but merely changes the kinetics of the induction of apoptosis cannot be excluded.

3.1.2 p53 induction following DNA damage.

Because of the different patterns of induction of apoptosis observed in ES cells, p53 protein expression was examined, by immunocytochemistry and western analysis, following γ and UVC-irradiation.

3.1.2.1 p53 induction following UVC-irradiation.

Following UVC ($37J/m^2$) treatment, the proportion of cells staining positively for p53 rose rapidly to a peak (88%) at 2 hours and returned to basal levels within 48 hours (Figure 16A). Western analysis revealed a similar rapid rise in p53 protein levels which peaked at 4 hours and again returned to basal levels within 48 hours (Figure 17). Mock-treated cells also showed a low amplitude, transitory p53 induction immediately after irradiation (Figure 16A). Cellular localisation of p53 was clearly nuclear (Figure 18).

3.1.2.2 p53 induction following γ-irradiation.

The response to γ-irradiation was more complex with two distinct phases of p53 expression (Figure 16B). Increased expression was observed, both in terms of the proportion of positively staining cells and absolute protein levels, within one hour of treatment and fell during the next seven hours (Figure 16B and 19). Mock-treated

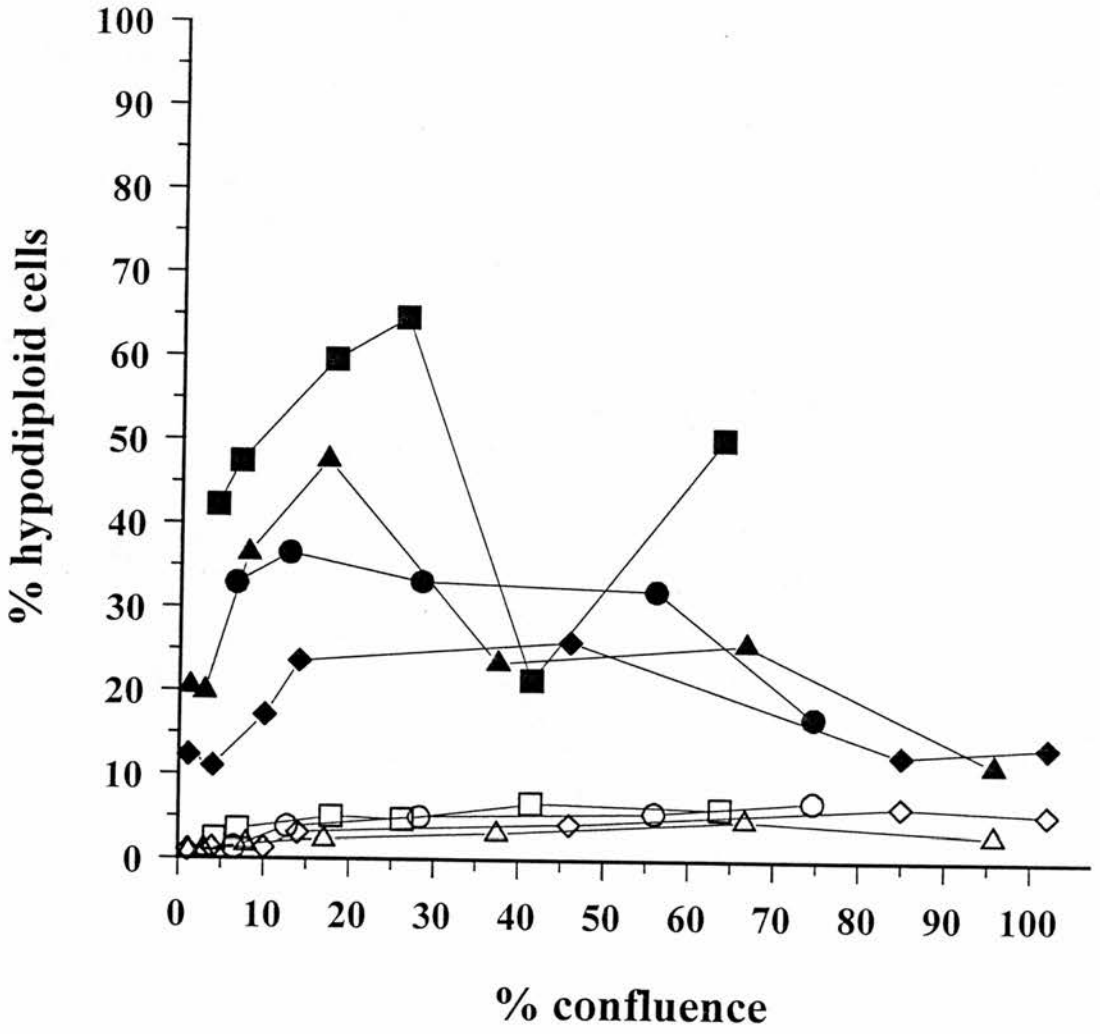


Figure 15: The effect of confluence levels of cell cultures prior to UVC-irradiation ($17J/m^2$) on the induction of apoptosis 24 hours after irradiation. Open symbols represent mock-irradiated controls and solid symbols irradiated cells. The experiment was repeated four times and each dataset is represented by a different symbol.

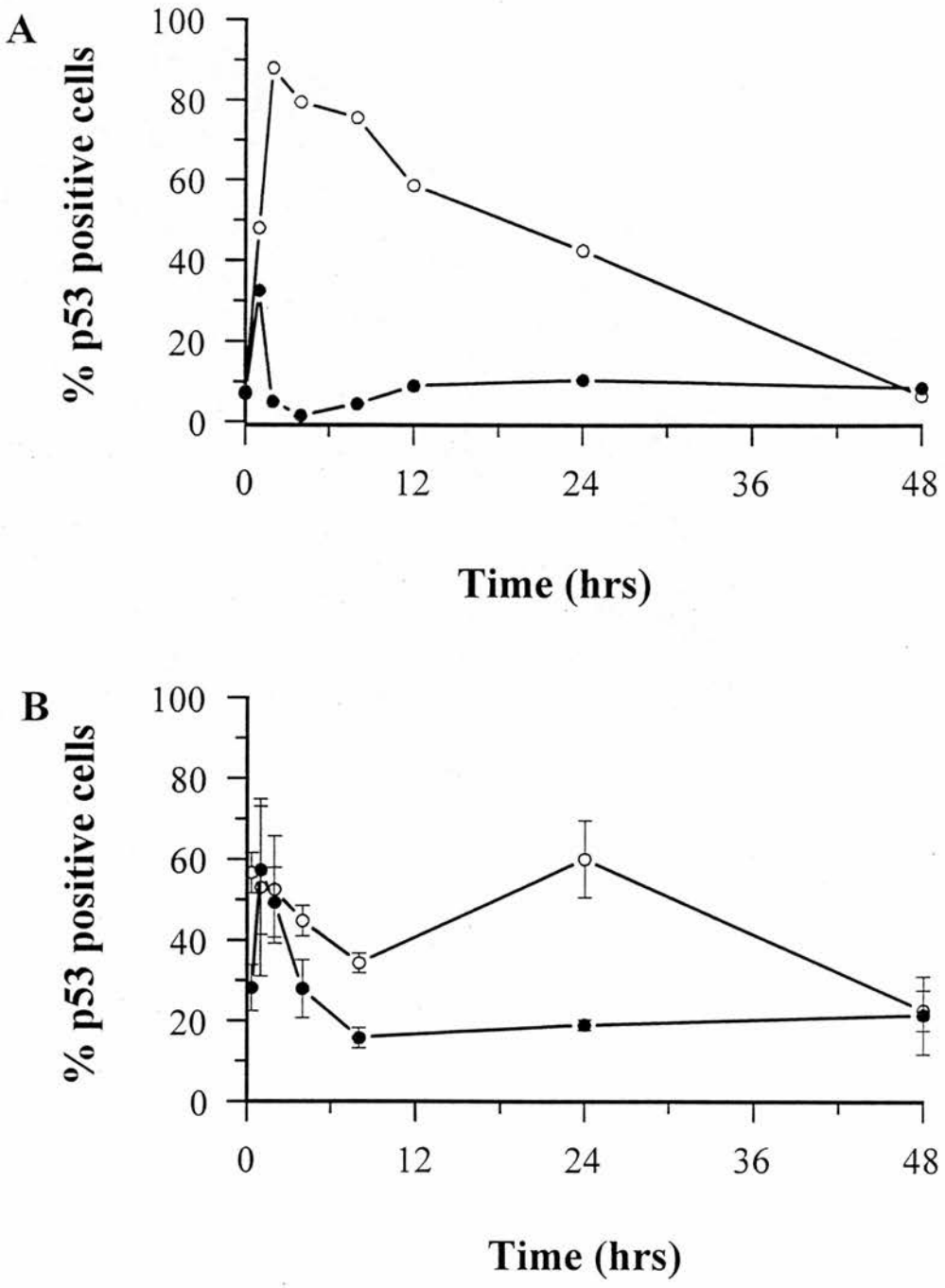


Figure 16: p53 induction in ES cells (E14) following 37J/m^2 UVC (A) or 7.6Gy γ-irradiation (B). Open symbols represent treated cells and solid symbols mock treated controls. In B, points represent the mean of four counts, two each derived from two separate time courses. Error bars represent the standard error.

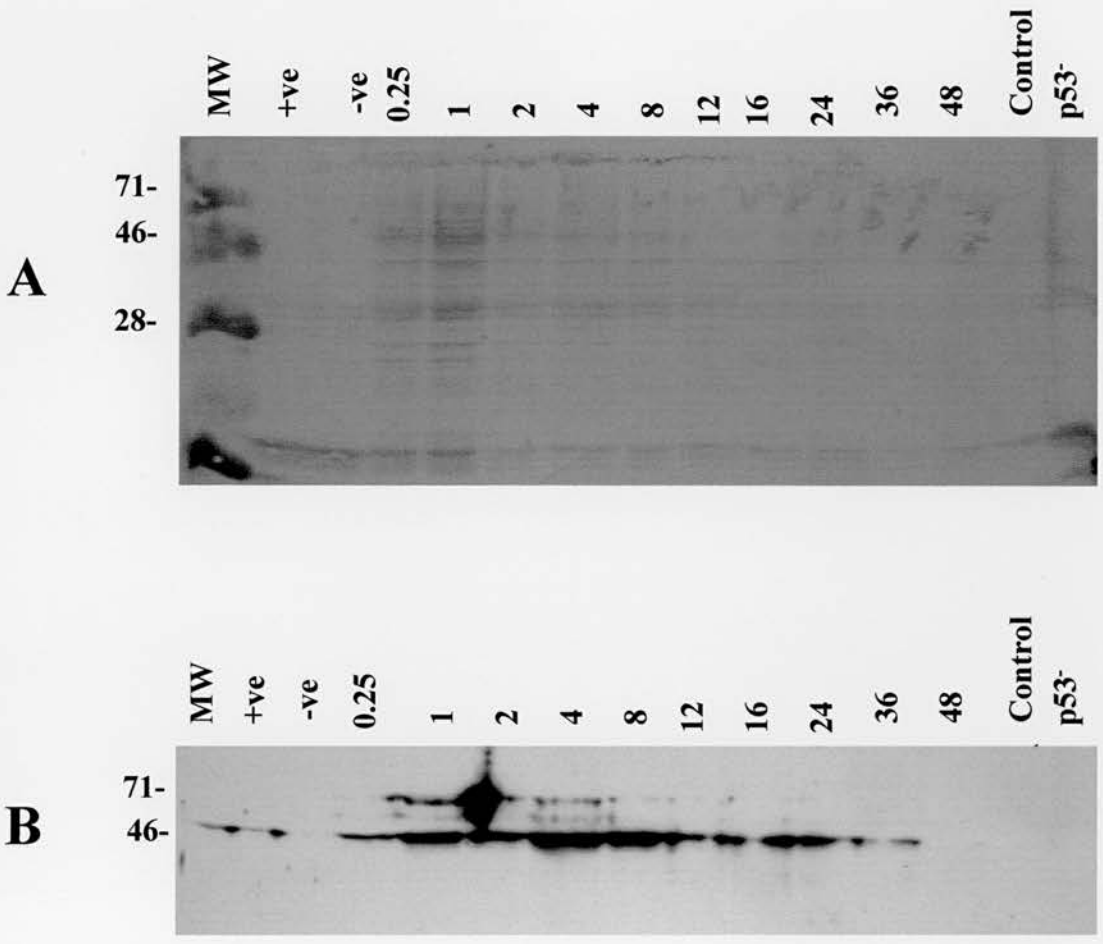


Figure 17: Western analysis of p53 expression in ES cells (E14) subsequent to treatment with UVC. The membrane was first labelled with Ponceau S stain to confirm uniform loading (A) and then labelled with the p53 antibody CM5 (B). MW indicates molecular weight markers. +ve indicates proteins extracted 7 hours subsequent to UVC irradiation ($50J/m^2$) as a positive control. -ve indicates proteins extracted from unirradiated cells. $p53^-$ indicates proteins extracted from p53 null cells as a negative control. Control indicates mock treated, unirradiated cells harvested 15 minutes after mock treatment. All other lanes contain proteins extracted from cells irradiated with $17J/m^2$ UVC and harvested at the timepoints indicated (in hours) above each lane. Sizes are indicated in kilodaltons at the side of each illustration.

A

B

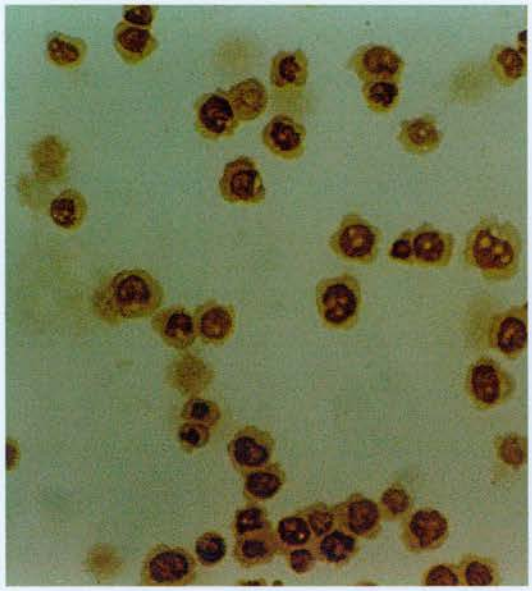


Figure 18: p53 immunocytochemistry following UVC-irradiation. Wild-type cells (E14) were stained with the polyclonal p53 antibody CM5. **A**: Mock treated control 2 hours after irradiation. **B**: Cells irradiated with 37J/m^2, 2 hours after treatment.

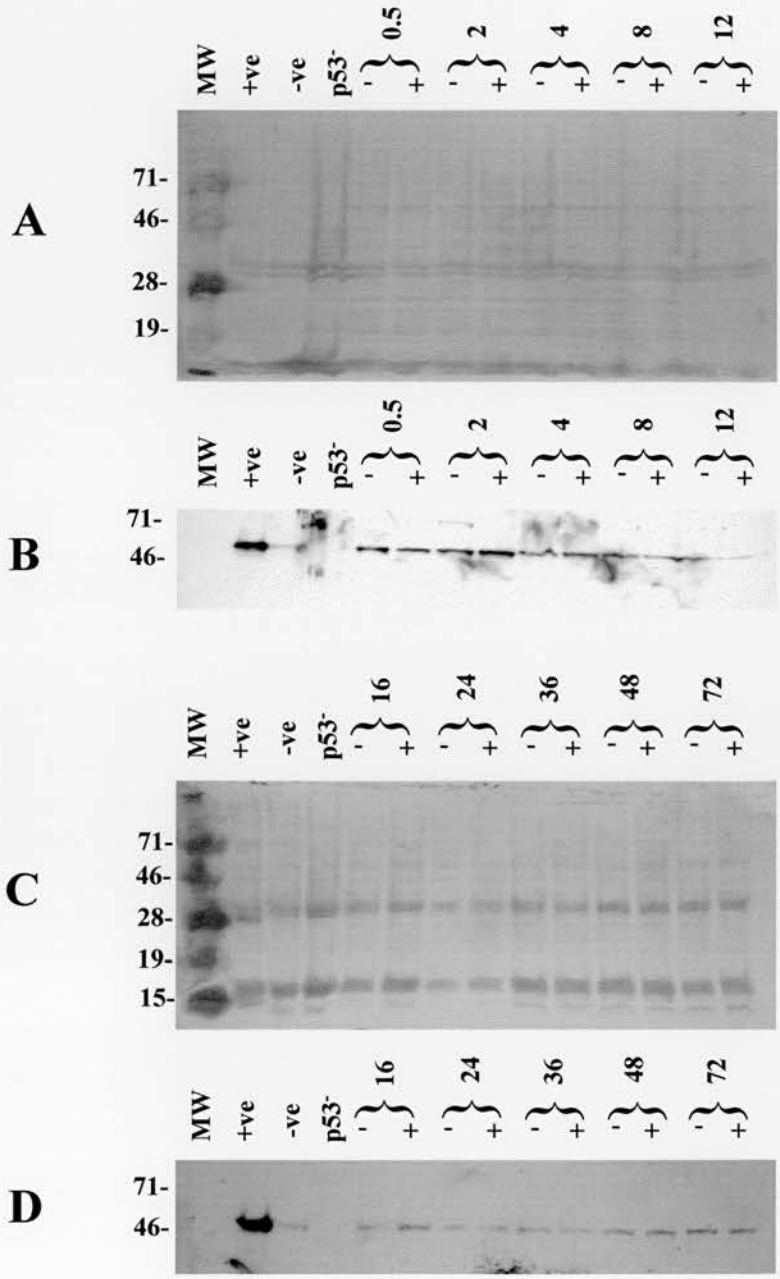


Figure 19: Western analysis of p53 expression in ES cells (E14) subsequent to treatment with ionising radiation (7.6Gy). Membranes were first labelled with Ponceau S stain to confirm uniform loading (A and C) and then labelled with the p53 antibody CM5 (B and D). MW indicates molecular weight markers. +ve indicates proteins extracted 7 hours subsequent to UVC-irradiation ($50J/m^2$) as a positive control. -ve indicates proteins extracted from unirradiated cells. $p53^-$ indicates proteins extracted from *p53* null cells as a negative control. + indicates cells irradiated with 7.6Gy ionising radiation and - mock-treated cells. Sizes are indicated in kilodaltons at the side of each illustration and time since irradiation (hours) above the relevant lanes.

cells also showed a transitory p53 induction immediately after γ-irradiation. Thus it is likely that the first phase of p53 expression observed in γ-irradiated cells is a consequence of stress due to handling. This explanation concurs with the fact that the exposure of the cells to γ-irradiation, which involved substantially more disruptions of culture conditions than did UVC-irradiation, resulted in a higher proportion of positive staining cells among the handling-controls. A second phase of increased expression was observed in the cytospins at 24 hours, with basal levels restored by 48 hours (Figure 16B). However this was not detectable in the western analysis, indicating no large, overall increase in protein levels (Figure 19).

One or more protein bands, of higher molecular weight than p53, were seen in the Western analysis after UVC-irradiation and, to a lesser extent, after γ-irradiation (Figures 17 and 19). The antibody used in this study (CM5) was polyclonal so it is possible that these bands represent either another member of the growing family of p53 homologues or perhaps a form of p53 that has undergone some type of secondary modification. However, the same Western blots labelled with a different polyclonal p53 antibody (Boehringer Mannheim Anti-p53-protein, pan (polyclonal BMG-1B1)) did not show these extra bands.

3.1.3 Cell cycle checkpoints following DNA-damage.

Flow cytometric analyses of cell cycle distribution following γ-irradiation revealed a G_2/M arrest. This arrest, which was not observed in unirradiated controls, was maximal within six hours of irradiation and remained elevated for at least 24 hours (Figure 20). Even 24 hours subsequent to irradiation, when the proportion of cells exhibiting G_2/M arrest was beginning to decline, the percentage of cells in G_2/M was significantly higher in irradiated than in unirradiated cells (χ^2 test, $p = 0.008$; Table 2). Like the induction of apoptosis after γ-irradiation, the proportion of cells in the G_2/M peak at 24 hours varied considerably between experiments. However, at this time point the proportion of cells in arrest was falling rapidly so that small changes in kinetics would result in large differences in the size of the G_2/M peak and this might

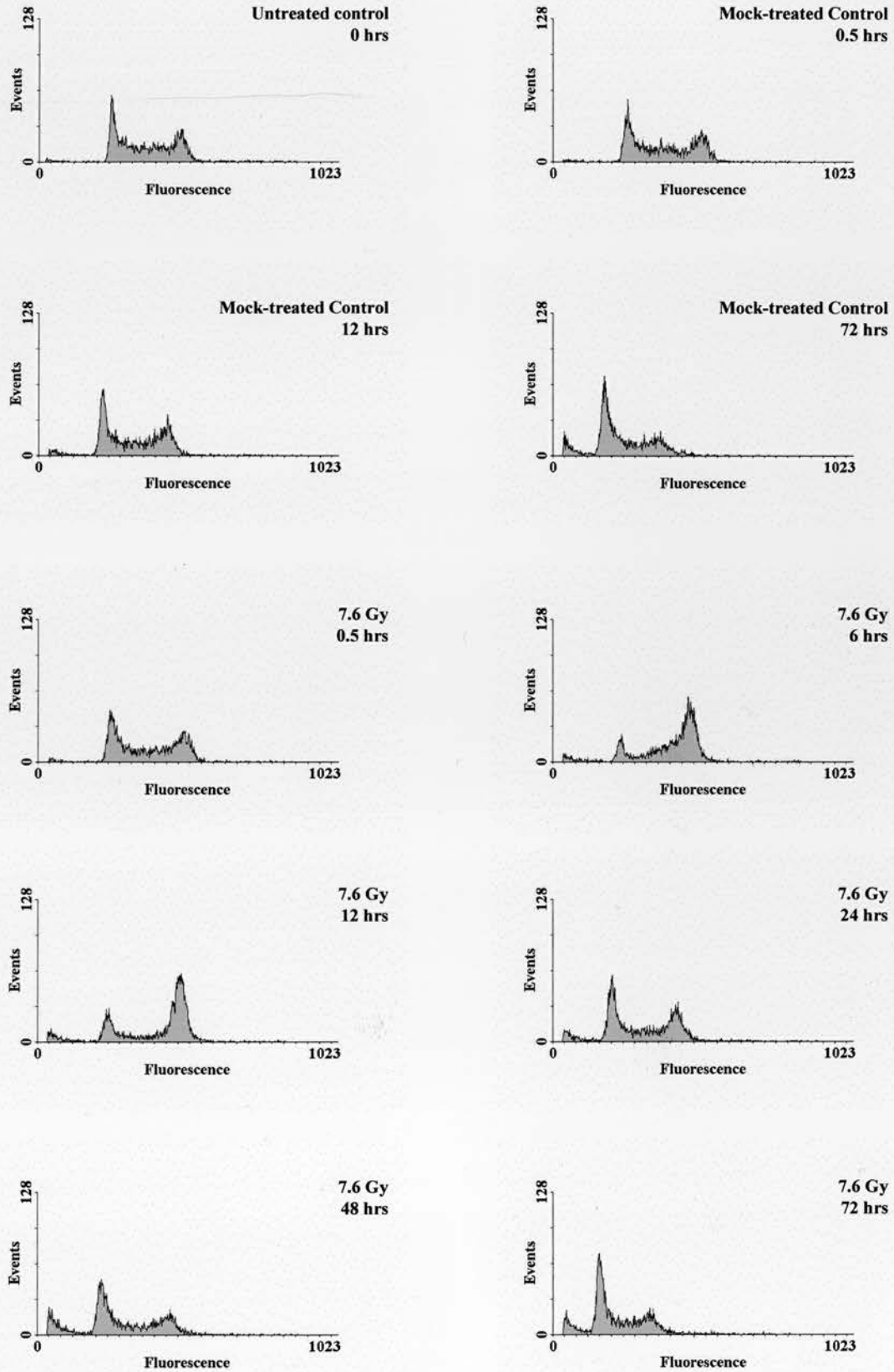


Figure 20: Flow cytometric analysis of G_2 arrest in wild-type ES cells (E14) following ionising radiation (7.6Gy). Radiation dose and time after irradiation are given in the top, right corner of each panel.

account for at least part of the observed variability. In agreement with previous experiments investigating the effects of γ-radiation on the induction of apoptosis, similar proportions of cells with a sub-G_1 DNA content were observed in irradiated and mock treated cells (Figure 20). In UVC-irradiated cells, cell cycle distribution did not differ between irradiated and mock treated cells.

Table 2: Comparison of the proportion of cells in G_2/M in irradiated (7.6Gy) and unirradiated cells 24 hours subsequent to treatment. Where two figures are given in a single column they represent two replicates.

Experiment	% G_2/M in unirradiated cells	% G_2/M in irradiated cells (7.6Gy)
1	19.5	20.8
2	22.6, 21.1	52.9, 32.8
3	22.1, 17.4	26.1, 50.9
4	20.2, 19.0	28.6, 26.2
Mean	20.27	34.04
Standard deviation	1.82	12.72

Summary.

In ES cells, UVC-irradiation and treatment with the topoisomerase inhibitors camptothecin and etoposide results in a rapid induction of apoptosis. In contrast, the apoptotic response to ionising radiation, even at high doses, was very weak and delayed relative to that observed following UVC-irradiation and topoisomerase inhibitors and, in some instances, did not differ significantly from controls. Following UVC-irradiation, rapid, nuclear stabilisation of p53 occurs several hours prior to the induction of apoptosis. Stabilisation of p53 after γ-irradiation was, like the apoptotic response, variable and late and was preceded by an early stress related response. ES cells do respond to γ-irradiation by a profound, extended G_2 arrest.

Section two:

The involvement of p53 in the apoptotic response of ES cells.

3.2.1 The derivation and analysis of *p53*-null ES cells.

Although the preceding experiments show p53 stabilisation occurs prior to apoptosis in ES cells, definitive proof that p53 is necessary for apoptosis can be derived only from experiments in which UVC treatment occurs in the absence of p53. To effect this, *p53*-null ES cell lines were derived by high G418 selection (Mortensen *et al.*, 1992) from a heterozygote cell line (R72) with a targeted inactivation of the *p53* gene (Clarke *et al.*, 1993).

3.2.1.1 The derivation and Southern analysis of *p53*-null ES cells.

The *p53*-null cell lines (R72D5, R72D27, R72D45 and R72D54) were derived from the heterozygous targeted cell line R72 (Clarke *et al.*, 1993) and were rendered homozygous by high dose selection at 1 (R72D5 and R72D27) or 2mg/ml (R72D45 and R72D54) of 100% active G418 (Mortensen *et al.*, 1992). Following selection, 63 resistant colonies (34 selected in 1mg/ml and 29 in 2mg/ml G418) were expanded into cell lines and screened by Southern blotting. DNA was extracted from these lines, digested with the restriction enzyme Bgl II and hybridised to a probe containing *p53* exons 10 and 11. Loss of the remaining, normal, *p53* allele would result in replacement of the wild-type 16kb fragment with an 11kb fragment (Figure 21; Clarke *et al.*, 1993). In the 1mg/ml experiment colonies had been very densely packed making it difficult to isolate monoclonaly derived resistant lines. Southern analysis of these lines revealed that almost half the colonies (15) were likely to have been polyclonal in origin (Figure 21). Only two of the remaining lines showed a restriction pattern consistent with a *p53*-null genotype whilst the remainder (17) displayed a restriction pattern not consistent with any of the expected genotypes (Figure 21). This pattern, which included the 16kb wild-type fragment and two additional bands of approximately 15kb and 8kb, was also observed at a high frequency (20 of 29 lines) in the second, 2mg/ml experiment. However in this

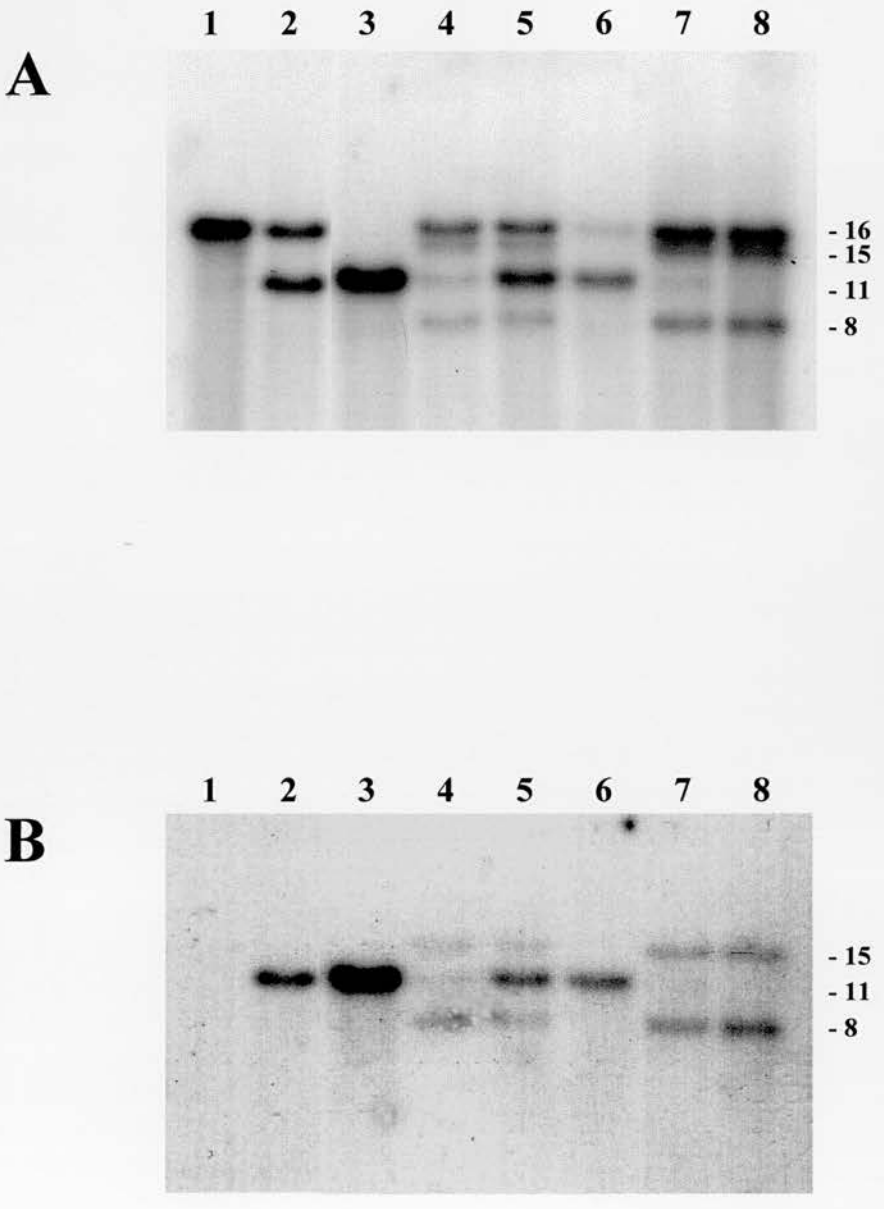


Figure 21: Southern analysis of R72 sub-lines selected in high levels of G418. 1, 129 mouse DNA (+/+); 2, R72 (+/-); 3, R72D54 (-/-); 4, 5, 6 and 7, sub-lines of polyclonal origin; 8, wild-type sub-line bearing a random integration of the *p53* targeting construct. A: Southern blot labelled with a probe containing exons 10 and 11 of the *p53* gene. B: The same Southern blot labelled with a probe for the neomycin resistance gene of the *p53* targeting construct. Sizes of bands (in Kb) are given to the right of each panel.

experiment very few selected lines (2) were of polyclonal origin and the proportion of *p53-null* lines was substantially higher (6). One possible explanation for the high frequency of lines showing the unexpected restriction fragment pattern would be that this pattern represents a *p53* wild-type line bearing randomly integrated copies of the *p53* targeting construct. The observed restriction fragment pattern is consistent with either the integration of two or more tandem copies of the plasmid at a single site or integration at two different sites. In either case, the line would have an enhanced resistance to G418, relative to the heterozygote, and if such a line were present as a contaminant at very low frequency in the original R72 cell line, selection in high levels of G418 would favour its growth. To establish whether or not this was the correct explanation, a Southern blot bearing examples of all of the observed restriction fragment patterns was hybridised to a probe for the neomycin resistance gene contained in the targeting construct. This probe would not be expected to bind to the largest wild-type fragment but would bind to the smaller, targeted band in *p53*-null and heterozygote lines and also the two bands derived from the targeting construct in the randomly integrated line. Consistent with the above explanation, no bands were observed in wild-type lines, one band was observed in *p53* heterozygote and null lines and two bands, one smaller and one larger than the *p53*-null band, were observed in the random integrant (Figure 21B). To further confirm the above hypothesis and as the heterozygote cell line R72 would be required in future experiments, R72 cells were subcloned and eight of the resulting clones genotyped by Southern blotting (Figure 22). Seven of the eight clones showed restriction fragment patterns consistent with heterozygosity at the *p53* locus whilst the eighth showed the pattern found in the random integrants observed in the two G418 selection experiments, thus confirming its presence as a contaminant in the original R72 cell line (Figure 22). *p53*-null cell lines to be used in future experiments were also subcloned to ensure a monoclonal origin.

3.2.1.2 Karyotypic analysis of *p53*-null ES cells.

Evidence from several sources suggests that loss of *p53* permits genomic instability and is commonly linked to aneuploidy in both normal and tumour cell types (see

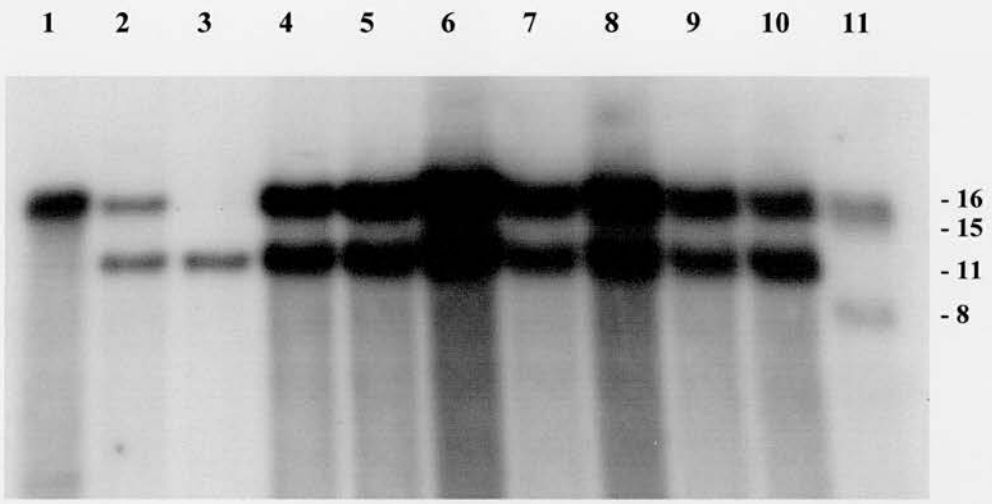


Figure 22: Southern analysis of R72 subclones labelled with a probe containing exons 10 and 11 of the *p53* gene. 1, 129 mouse DNA (+/+); 2, R72 (+/-); 3, R72D5 (-/-); 4, 5, 6, 7, 8, 9 and 10, R72 subclones showing the R72 restriction fragment pattern; 11, wild-type subclone bearing a random integration of the *p53* targeting construct. Sizes of bands (in Kb) are given to the right of the panel.

section 1.3.6). If the *p53*-null cells derived here contained chromosomal abnormalities, this would obviously have implications for the interpretation of any data obtained from them. Thus, the karyotypic stability of *p53*-null stem cell lines and their parental heterozygote and wild-type cell lines was tested over 20 passages in culture (approximately ten weeks). At the lowest passages analysed (five to nine passages after derivation) all four null cell lines had an abnormal karyotype, three having a modal near-diploid number of 41 (R72D5, R72D27 and R72D54) whilst one was aneuploid (R72D45), having 67 chromosomes (Figures 23 and 24). However, in none of the clones did this reflect continuing karyotypic instability as no further divergence was observed over up to 15 additional passages (Figures 23 and 24). The parental cell line (E14) was found to have a diploid modal chromosome number but also had an unexpectedly high proportion of metaphase spreads having a near-diploid number of 41. This karyotype was also maintained over 20 additional passages. Fluorescent *in situ* hybridisation, using a chromosome 11 paint, revealed that the abnormal karyotypes of R72D5, R72D27 and R72D54 were the result of trisomy of chromosome 11 (Figure 25 and Table 3). The murine *p53* gene is located on chromosome 11, hence this result confirms the reduplication of the targeted chromosome in the neomycin resistant cells. Trisomy of chromosome 11 was also observed in a proportion of metaphase spreads from E14s at a level that would be consistent with this abnormality being the cause of the additional chromosome seen in some spreads (Table 3).

3.2.2 The apoptotic response in *p53*-null ES cells.

To assess the requirement for p53 in ES cell apoptosis, wild-type and *p53*-null cells were exposed to both etoposide and UVC-irradiation.

3.2.2.1 The apoptotic response to Etoposide in *p53*-null cells.

Wild-type (E14) and *p53-null* (R72D54) ES cells were exposed (in triplicate) to doses of etoposide ranging from 0 to 20μM and harvested 26 hours later for scoring of apoptosis after acridine orange staining. Levels of apoptosis in wild-type cells

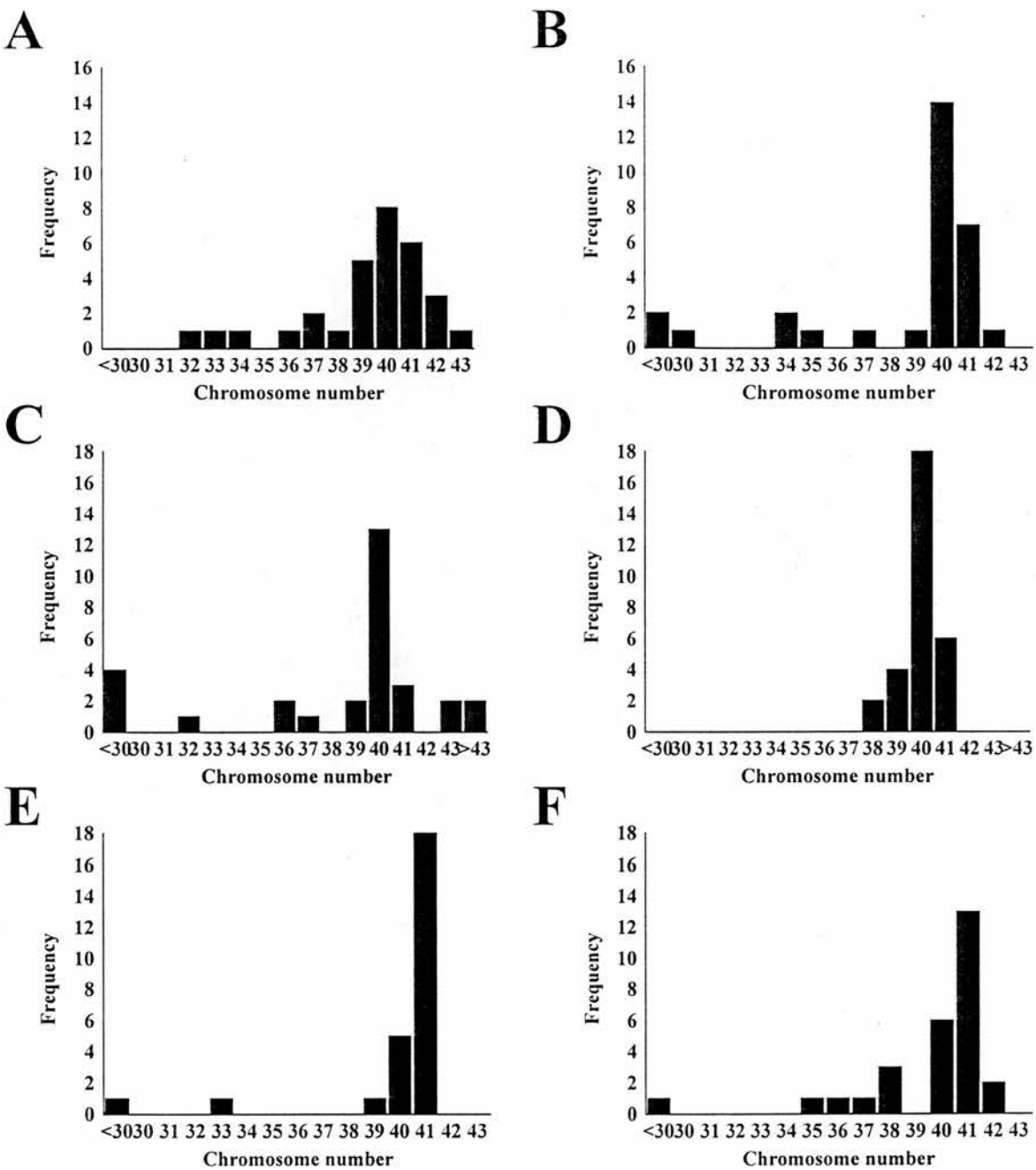


Figure 23: Karyotypic analysis of wild-type, heterozygous and *p53* null ES cell lines. A: E14 (+/+) cell line at a passage equivalent to that of the *p53* null cell lines five passages after derivation. B: E14 cell line at a passage equivalent to that of the *p53* null cell lines 20 passages after derivation. C: R72 (+/-) cell line at a passage equivalent to that of the *p53* null cell lines five passages after derivation. D: R72 cell line at a passage equivalent to that of the *p53* null cell lines 20 passages after derivation. E: R72D5 (-/-) cell line five passages after derivation. F: R72D5 cell line 20 passages after derivation.

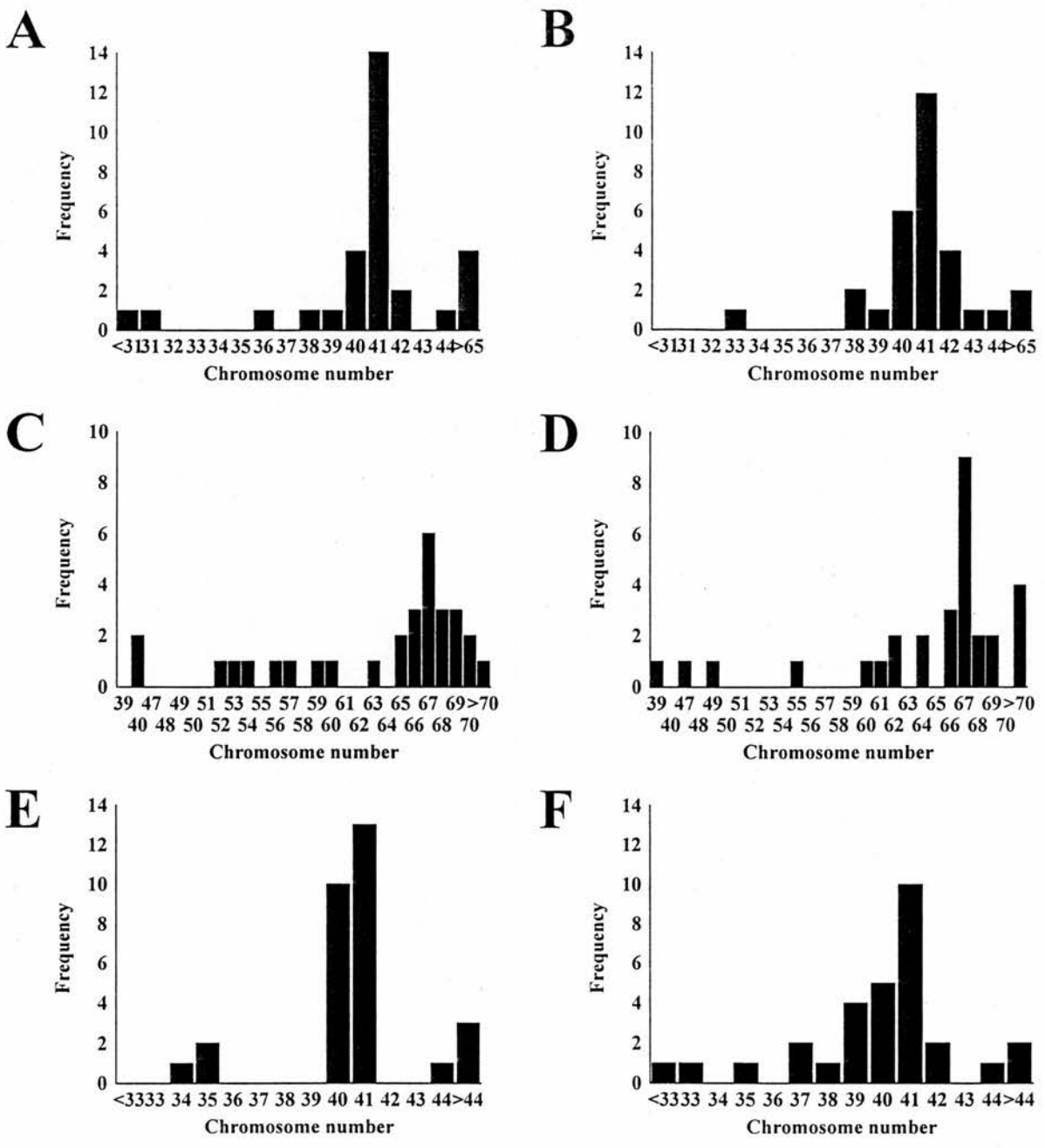


Figure 24: Karyotypic analysis of *p53* null ES cell lines. A: R72D27 cell line six passages after derivation. B: R72D27 cell line 20 passages after derivation. C: R72D45 cell line five passages after derivation. D: R72D45 cell line 20 passages after derivation. E: R72D54 cell line nine passages after derivation. F: R72D54 cell line 26 passages after derivation.

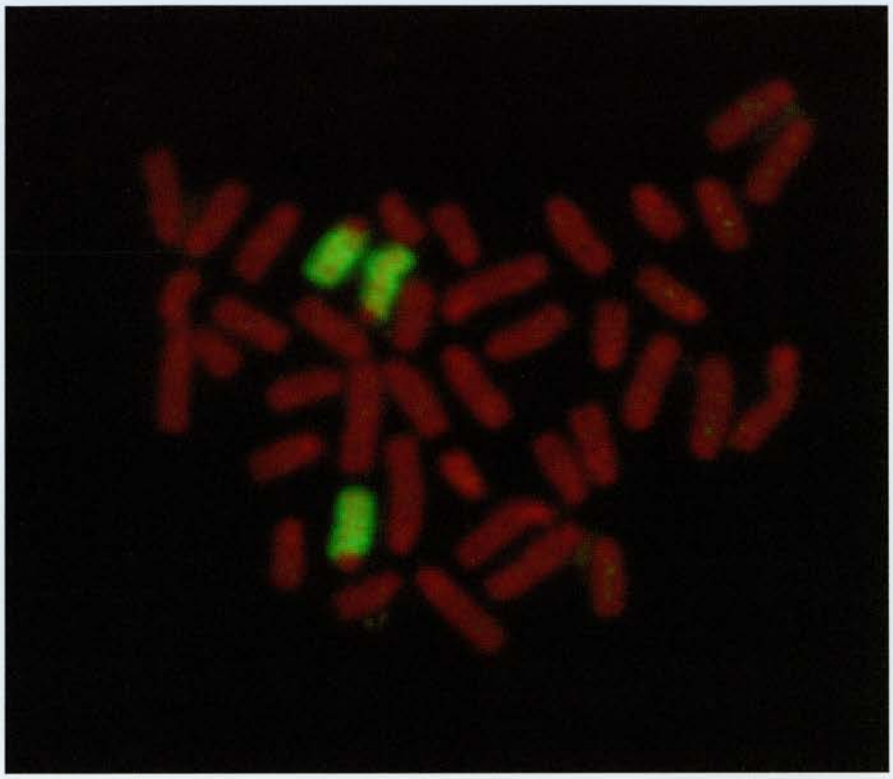


Figure 25: Fluorescent *in situ* hybridisation of a murine chromosome 11 paint showing trisomy of chromosome 11 in the *p53* null ES cell line R72D5 (1000x magnification).

Table 3:. The frequency of trisomy of chromosome 11 in wild-type, heterozygote and *p53*-null cell lines. For each cell line one hundred metaphase spreads were scored for chromosome 11 trisomy.

Cell line.	**Passage.**	**% of cells showing trisomy.**
E14	5*	16%
R72	5*	4%
R72D5	5	84%
R72D27	6	79%
R72D54	9	59%

- For clarity, passage numbers of parental cell lines are given relative to the time at which *p53-null* cells were derived from them, not since the time of their own derivation.

were consistent with previous results (see section 3.1.1.2) and showed a clear, dose-dependent increase in apoptosis (one-way ANOVA, p=0.000; Figure 26). In *p53-null* cells, there was no induction of apoptosis, at this time-point, after exposure to the two lower doses of etoposide (0.2 and 2μM). However, exposure to 20μM etoposide did result in levels of apoptosis significantly higher than those observed in control cultures (t-test, p=0.025). This partial p53-dependence of the apoptotic response to etoposide is consistent with results previously obtained from thymocytes (Clarke *et al.*, 1993).

3.2.2.2 The apoptotic response to UVC-irradiation in *p53*-null cells.

Three wild-type, p53 heterozygous and p53-null cell lines were exposed (in triplicate) to 93J/m^2 UVC and harvested for acridine orange counts after 16 hours. Induction of apoptosis was clearly p53 dependent at this time-point; it showed near background levels in *p53*-null cells and an intermediate response in *p53* heterozygotes (Figure 27). A qualitatively similar response was seen 24 hours after a lower (37J/m^2) UVC dose (Figure 28). However, *p53*-null cells still exhibited low but significant levels of apoptosis in response to UVC indicating that p53-independent apoptotic pathways exist in these cells (ANOVA: 93J/m^2, P=0.006). The level of apoptosis observed in wild-type cells at the higher UVC dose was less than anticipated from previous experiments. This may reflect variations in the kinetics of induction between experiments or variations between experiments in the levels of apoptosis induced.

3.2.3 Cell cycle arrest in *p53*-null ES cells.

Previous experiments on cell cycle distribution of wild-type ES cells following γ-irradiation had revealed a substantial G_2/M arrest peaking around six hours after treatment (see section 3.1.3 and Figure 20). A similar investigation in *p53*-null ES cells revealed that this arrest was p53 independent. As in wild-type cells, this arrest was maximal within six hours of irradiation and remained elevated for at least 24 hours (Figure 29). A small proportion of cells with a sub-G_1 DNA content was

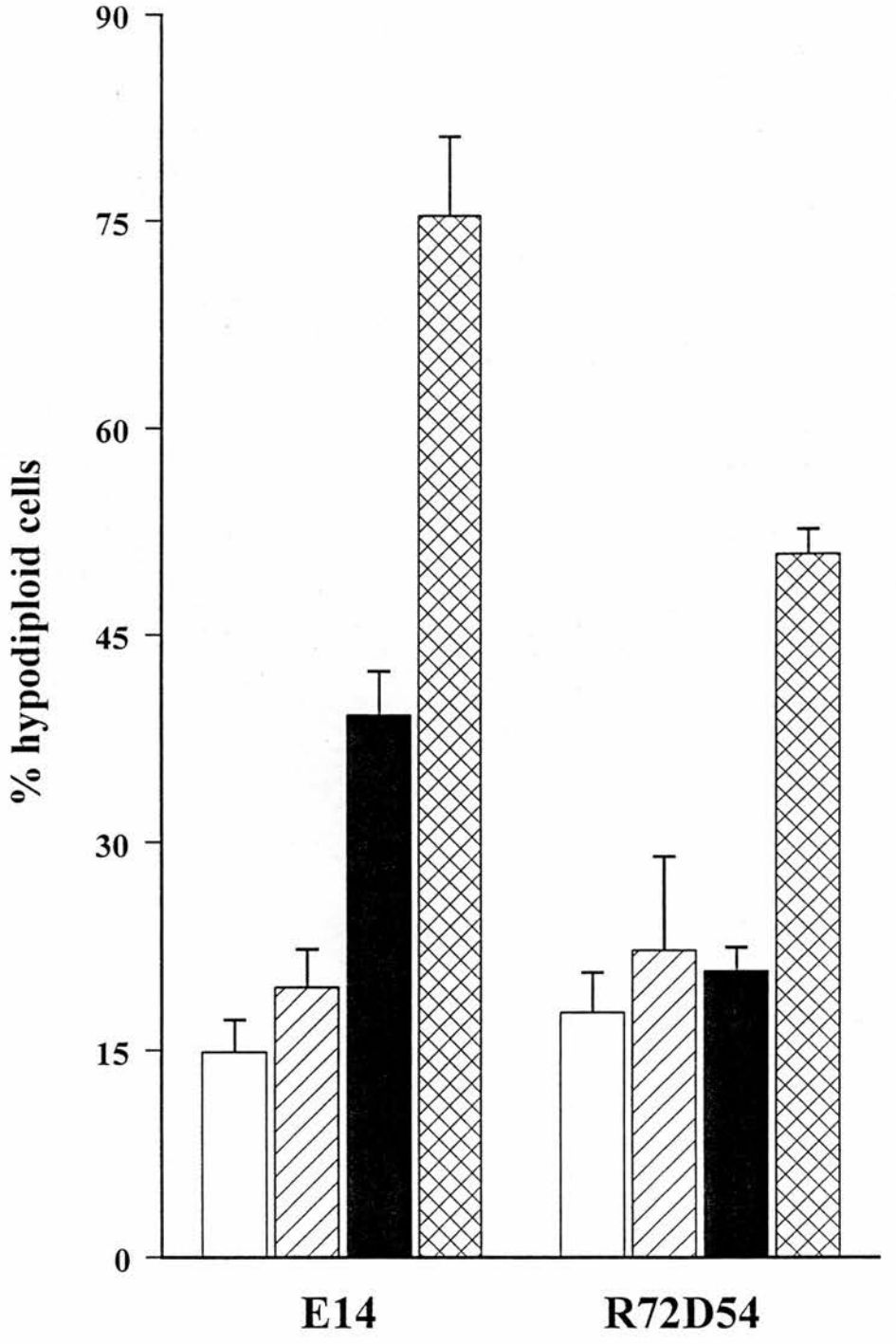


Figure 26: Induction of apoptosis in wild-type (E14) and *p53* null (R72D54) cells 26 hours after treatment with etoposide. Open bars, mock-treated controls; hatched bars, 0.2μM etoposide; solid bars, 2μM etoposide; cross-hatched bars, 20μM etoposide. Error bars represent the standard error.

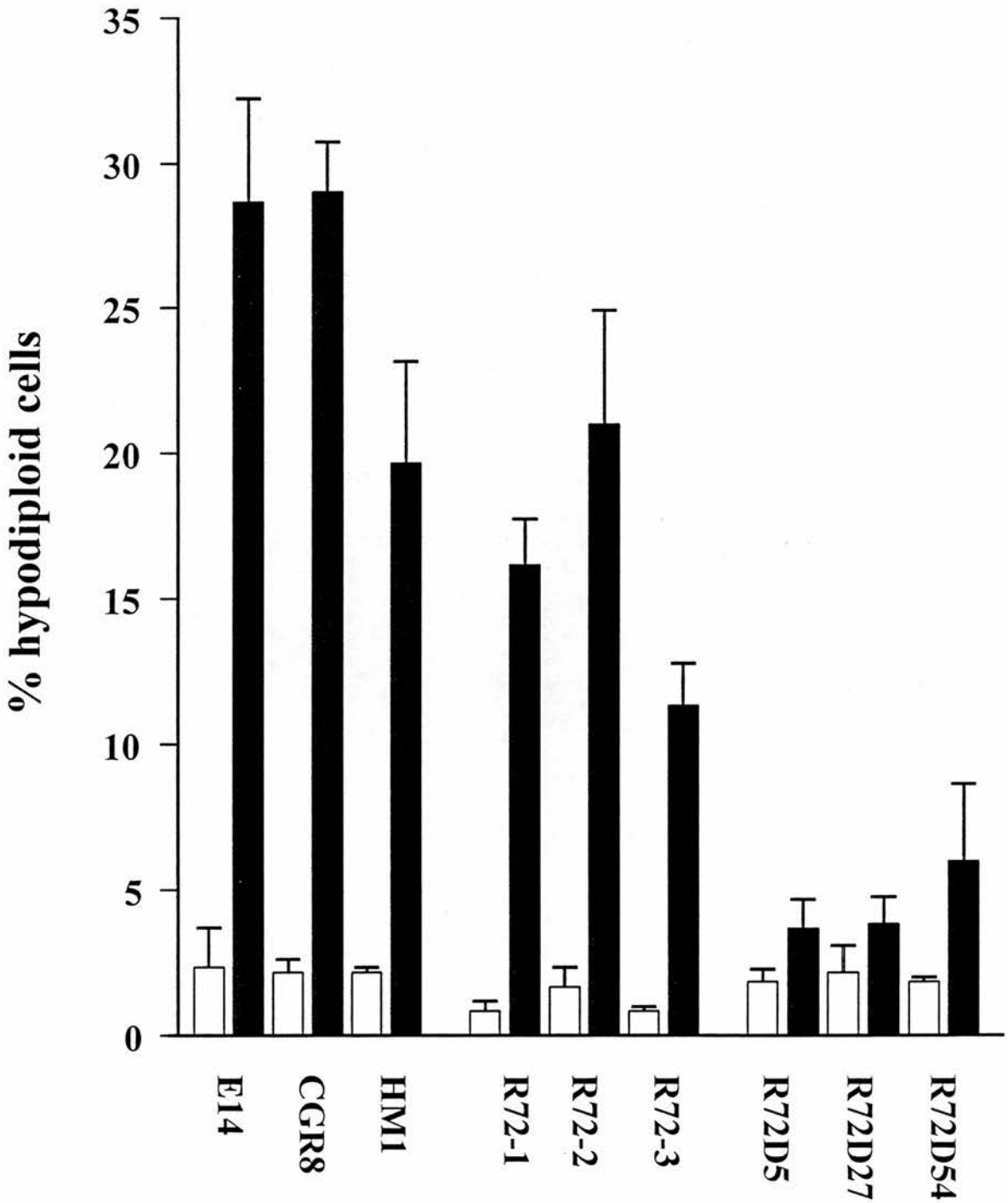


Figure 27: Induction of apoptosis 16 hours subsequent to exposure to UVC in three independent wild-type (E14, CGR8 and HM1), *p53* hemizygous (R72-1, R72-2 and R72-3) and null cell lines (R72D5, R72D27 and R72D54). Open bars, mock-treated controls; solid bars, cells treated with 93J/m^2. Each bar represents the mean of three replicates and error bars give the standard error.

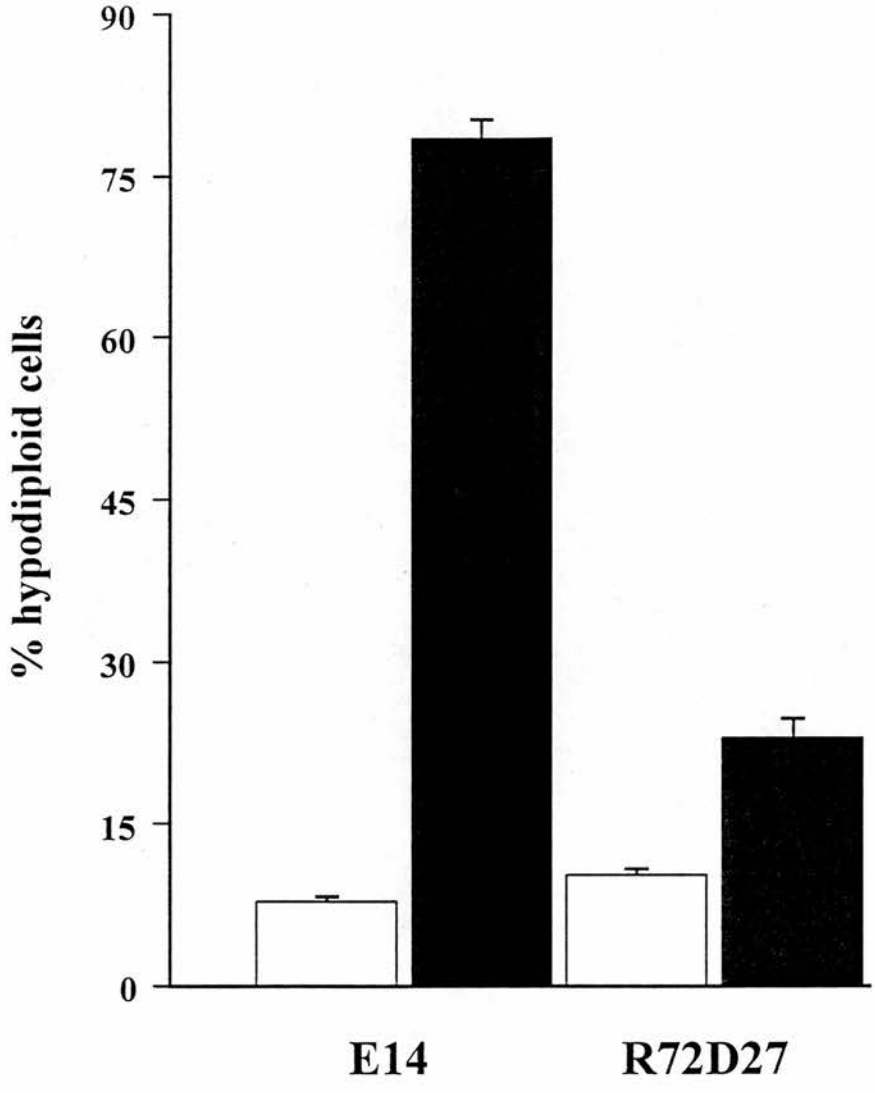


Figure 28: Induction of apoptosis 24 hours subsequent to exposure to UVC in wild-type (E14) *p53* null cell lines (R72D27). Open bars, mock-treated controls; solid bars, cells treated with $17J/m^2$. Each bar represents the mean of three replicates and error bars give the standard error.

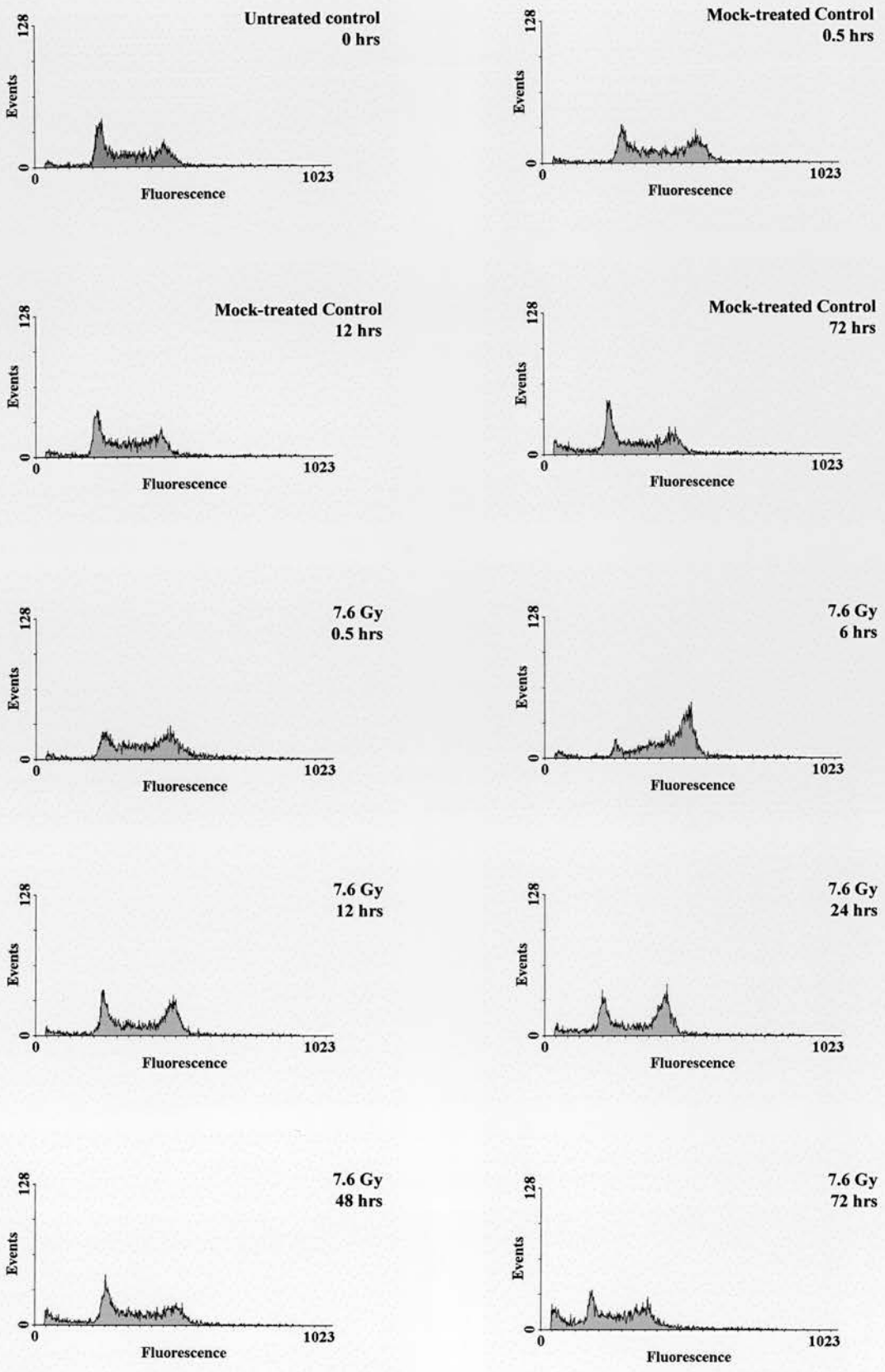


Figure 29: Flow cytometric analysis of G_2/M arrest in *p53*-null ES cells (R72D5) following ionising radiation (7.6Gy). Radiation dose and time after irradiation are given in the top, right corner of each panel.

observed in both irradiated and mock treated cultures at later time-points. A comparison of the proportion of cells in the G_2/M peak in untreated and mock-treated controls suggests that a small G_2/M arrest may also have occurred in the mock-treated controls. A similar though smaller increase was observed in wild-type cells (Figure 20) but in both cases it had been resolved by 12 hours (Figures 20 and 29). This possible arrest was not investigated further but such a handling-induced arrest would not be surprising considering the stress induced induction of p53 observed after mock-irradiation. A comparison of the cell cycle distribution between wild-type (Figure 20) and *p53*-null ES cells (Figure 29) suggests that loss of p53 might also affect the kinetics of the cell cycle.

3.2.4 Summary.

p53 is required for the rapid induction of apoptosis seen in ES cells after etoposide and UVC-irradiation. However, both etoposide and UVC induce p53 independent apoptosis. Chromosomal stability was maintained in three out of four of the *p53*-null cell lines derived from heterozygotes by high dose G418 treatment. However, one of the cell lines was found to be highly aneuploid suggesting the involvement of other causative factors.

Section three:
The long term consequences of DNA damage.

3.3.1 Clonogenic survival subsequent to DNA damage.

Evasion of apoptosis in the short term does not necessarily imply a continued capacity for growth. Therefore clonogenic survival of wild-type and *p53*-null ES cells was examined following UVC and γ-irradiation.

3.3.1.1 Clonogenic survival following UVC-irradiation.

Cells were exposed to UVC doses ranging from 0 to 50J/m^2. Plating efficiency did not differ between *p53*-null and wild-type cells (t-test; df= 69, p=0.89). In both wild-type and *p53*-null cells survival was greatly reduced, in a dose-dependent manner, following UVC-irradiation (Figure 30A). However, survival of *p53*-null cells was consistently higher than that of wild-type cells (Friedman test; p = 0.001).

3.3.1.2 Clonogenic survival following γ-irradiation.

After γ-irradiation at doses ranging from 0 to 19Gy, the clonogenic survival of both wild-type and *p53*-null cells was reduced in a dose-dependent manner (Figure 30B). In wild-type cells, a UVC dose of 10J/m^2 and a γ-irradiation dose of 3.8Gy resulted in similar levels (around 10%) of survival. Again, survival of *p53*-null cells was consistently higher than that of wild-type cells (Friedman test; p = 0.014).

3.3.2 *Hprt* mutation frequencies of wild-type and *p53*-null ES cells.

Even at the highest γ-irradiation dose of 19Gy, up to 1.4% of wild-type and 14.4% of *p53*-null cells survived to form colonies (Figure 30B). It has been predicted that a *p53*-null environment will result in an increased mutation frequency due to loss of the normal apoptotic pathway and subsequent survival of cells bearing DNA damage. This prompted us to investigate the mutation frequency at the *Hprt* locus after UVC and γ-irradiation. The assay used for this purpose measures the proportion of those cells that survive irradiation which bear mutations in the *Hprt* gene, but does not assess the effects of irradiation on cell survival.

Following UVC-irradiation, a large dose-dependent increase in mutation frequency, up to 100-fold at higher doses, was observed. Surprisingly, the frequency in *p53*-null cells was only two-fold higher than that in wild-type cells, although this difference was significant (Sign test, p = 0.03; Figure 31). In contrast, subsequent to γ-irradiation a less than 10-fold increase in mutation frequency was detected with no significant difference between wild-type and *p53*-null cells (Figure 31).

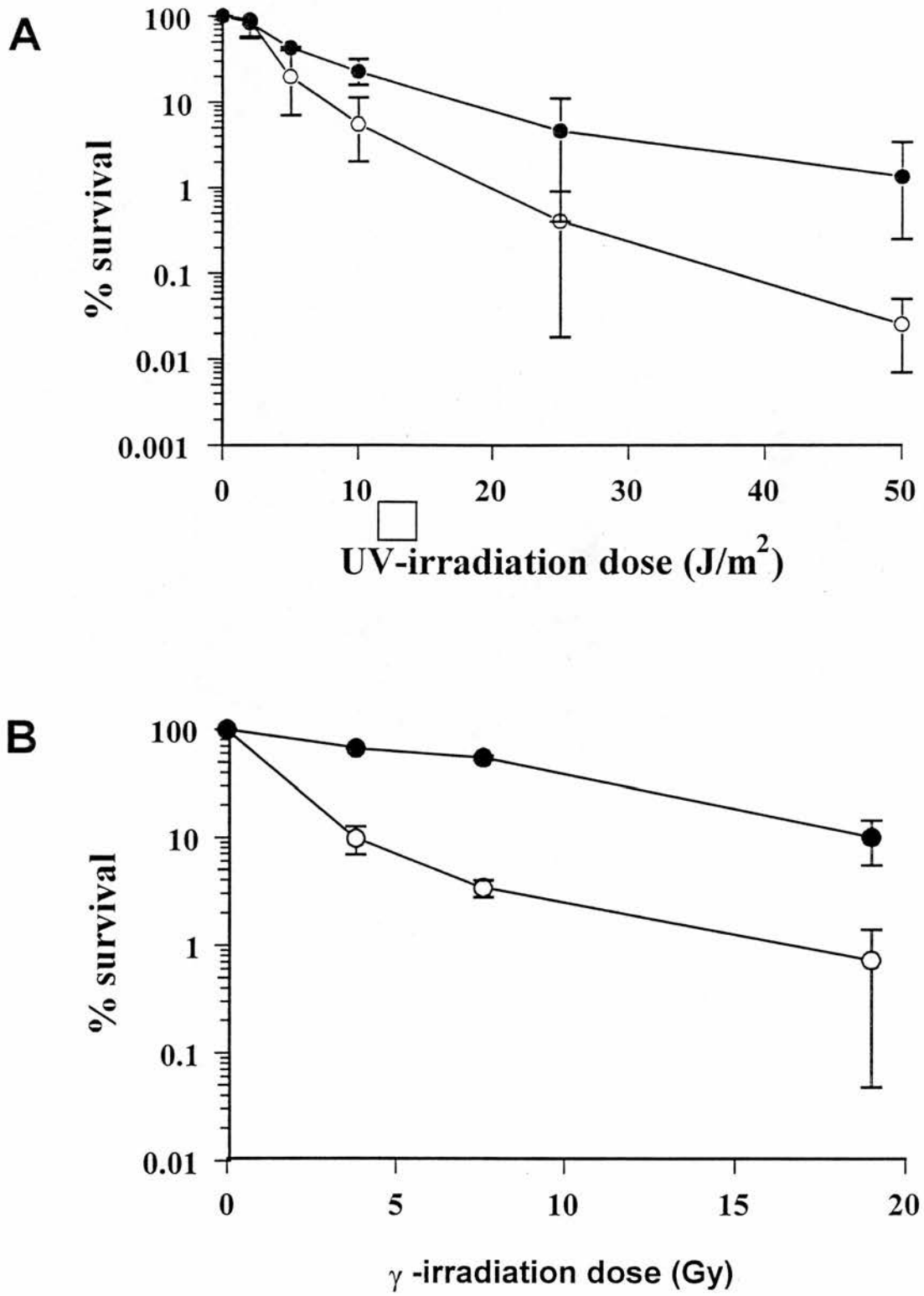


Figure 30: Clonogenic survival of wild-type (E14, open symbols) and *p53* null (R72D5, solid symbols) cells following UVC-irradiation (A) and γ-irradiation (B). Data points represent the mean of two experiments for γ-irradiation and three experiments for UVC-irradiation. Error bars indicate the range.

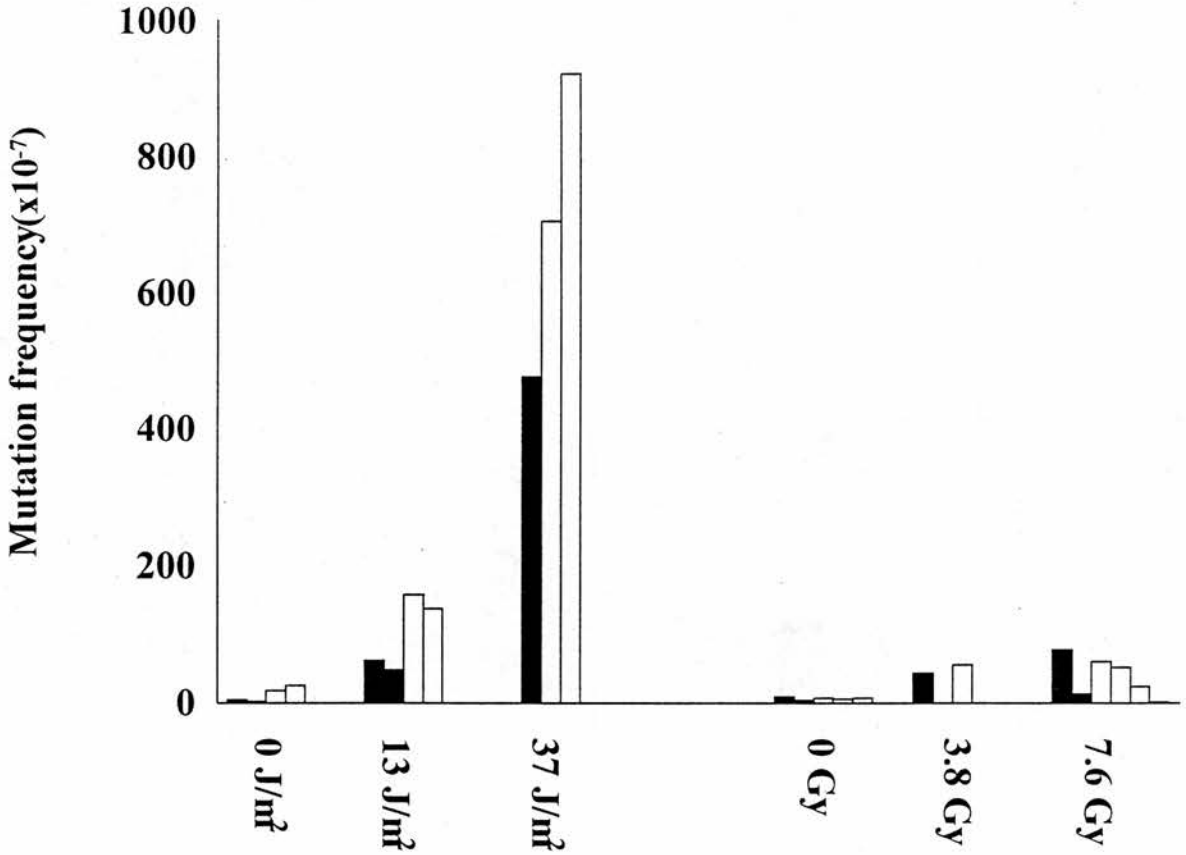


Figure 31: *Hprt* mutation frequencies of wild-type (E14, solid bars) and *p53* null (R72D5, open bars) cells subsequent to UVC-irradiation and γ-irradiation. Each bar represents a separate experiment.

3.3.3 The *Hprt* mutation spectrum in p53-null ES cells following γ-irradiation.

One potential source of error in the type of mutation assay used in this study is that the mutation frequency may be overestimated due to the overgrowth of a small proportion of clones. To address this issue and to obtain a more detailed picture of the mutagenic events, the clonal origins of 22 6-TG resistant, *p53*-null colonies were investigated. At the completion of the *Hprt* assay, colonies which had been exposed to 7.6Gy γ-radiation were analysed by PCR and Southern blotting.

Two PCRs were performed on cDNA samples from each clone, the first amplifying the entire coding sequence of the *Hprt* gene and the second a control reaction amplifying a 1265 bp region of the constitutively expressed *Tbp1* gene. Of the 22 clones analysed, all produced *Tbp1* PCR products whilst only eight produced *Hprt* products (Table 4 and Figure 32). When the *Hprt* PCR products of these colonies were sequenced, all eight were found to have distinctive mutations. These included six deletions, ranging in size from three to 290 bp, one transition and one transversion (Table 4). One of the clones (H12) produced two PCR products, the first with a deletion of exons two and three and the other with only exon three deleted, suggesting a mutation affecting the splicing machinery of the gene.

Where possible, clones were also analysed by southern blotting and hybridisation to an *Hprt* cDNA probe. Restriction digest of the *Hprt* gene with the enzyme EcoRI results in five fragments varying in size from 1.3 to 9.3kb (Thompson *et al.*, 1989; Figure 33A). An additional band, running between the 5.0 and 1.3kb bands, is often seen and is derived from the pseudogene. Of those clones which had been sequenced, all gave EcoRI restriction digest patterns which were consistent with their known cDNA mutations (Tables 4 and 5, Figure 33). Of the remaining 12 clones analysed, nine showed no labelled bands at all indicating deletion of the entire *Hprt* gene, one had a deletion removing one of the primer sequences and two had lost none of the expected bands suggesting either small changes in the PCR primer region or loss of upstream control sequences (Table 5, Figure 33). The spectrum of mutations observed were thus consistent with the type of mutations expected

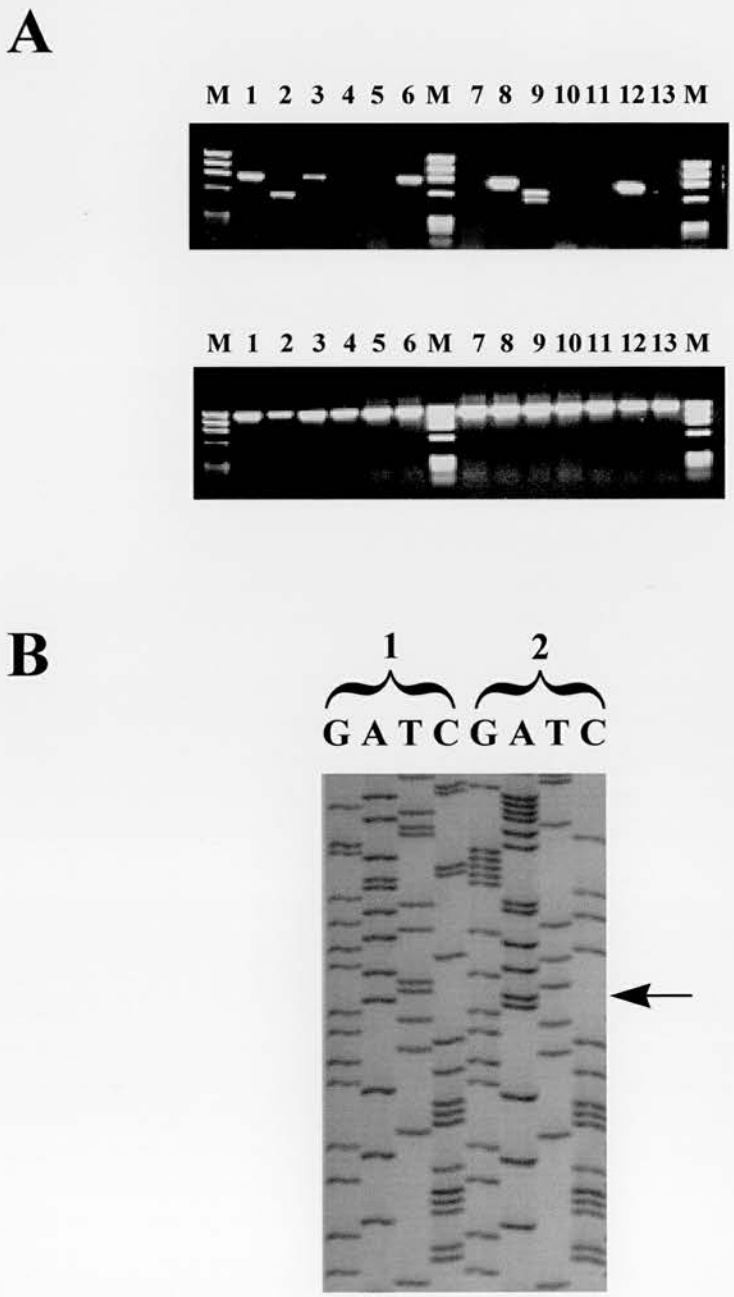


Figure 32: Typical examples of PCR (A) and sequencing (B) analyses of mutant *Hprt* clones. A: Upper panel, HPRT PCR reactions; lower panel, TPB1 PCR reactions. M, ΦX174 molecular weight marker (sizes of the upper four bands are 1.4, 1.1, 0.9 and 0.6kb); 1, E14 control reaction; 2, H23; 3, H21; 4, H31; 5, H27; 6, H28; 7, H11; 8, H10; 9, HH12; 10, H30; 11, H17; 12, H29; 13, H34. B: Sequence of wild-type (1) and mutant (2) *Hprt* PCR products. The arrow marks the beginning of the deletion in clone H23.

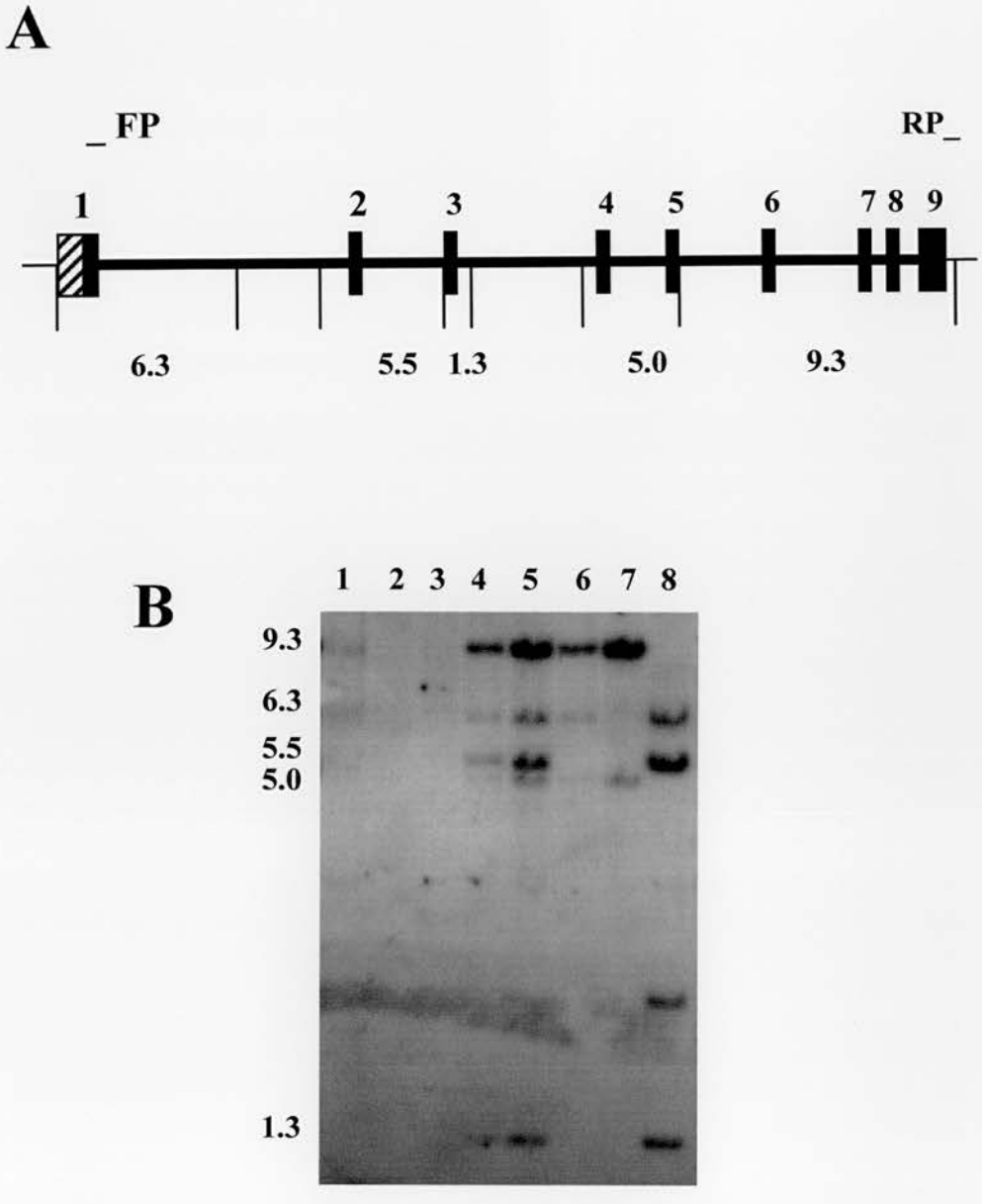


Figure 33: **A**: Structure of the wild-type *Hprt* gene (reproduced from Thompson *et al.*, 1989). Closed boxes, endogenous exons; thick lines, endogenous introns; thin horizontal lines, flanking sequence; cross-hatched boxes, promoter regions; thin vertical lines, EcoRI sites. The number of each exon is shown above it and the size (kb) of EcoRI fragments shown below and between sites. FP shows position of forward primer and RP reverse primer. **B**: Typical example of a Southern analysis of *Hprt* mutant clones. Clones were digested with the restriction enzyme EcoRI and hybridised to an *Hprt* cDNA probe. Sizes of expected restriction fragments are given (in kb) to the left of the panel. Lane 1, H16; 2, H17; 3, H18; 4, H19; 5, wild-type pattern; 6, H23; 7, H24; 8, H26.

Table 4: Mutations detected in the cDNA of the *Hprt* gene of eight *Hprt* deficient ES cell clones. Positions of mutations are given according to Konecki *et al.* (1982).

Clone	Mutation	Position	Effect
H10	3 bp deletion	165 to 167 bp	Deletion of one amino acid (His)
H12	182 and 290 bp deletion	224 to 406 and 116 to 406 bp	Exon 3 deleted Exon 2 and 3 deleted
H19	47 bp deletion	573 to 619 bp	Frameshift at 573 bp and stop at 632 bp
H21	C to G	455 bp	Stop at 455 bp
H23	290 bp deletion	116 to 406 bp	Exon 2 and 3 deleted
H26	246 bp deletion	449 to 695 bp	Deletion of part of exon 3 and exons 4 to 8
H28	G to T	604 bp	Stop at 604 bp
H29	39 bp deletion	60 to 99 bp	Frameshift at 99 bp and stop at 115 bp

Table 5: Summary of EcoRI band patterns observed in *Hprt* mutants when hybridised to *Hprt* cDNA. Sizes of the expected wild-type bands are given at the head of each column. + indicates presence of the band, - indicates absence and R the presence of a band reduced in size.

Clone	9.3kb	6.3kb	5.5kb	5.0kb	1.3kb
H10	+	+	+	+	+
H11	+	+	+	+	+
H14	-	-	-	-	-
H16	-	-	-	-	-
H17	-	-	-	-	-
H18	-	-	-	-	-
H19	R	+	+	+	+
H20	-	-	-	-	-
H21	+	+	+	+	+
H23	+	+	-	+	-
H24	+	-	-	+	-
H26	-	+	+	R	+
H27	+	+	+	+	+
H28	+	+	+	+	+
H30	-	-	-	-	-
H31	-	-	-	-	-
H33	-	-	-	-	-
H34	-	-	-	-	-

subsequent to this type of DNA damage (reviewed by Sankaranarayanan, 1991). Although it was not possible to examine the clonal origins of all *Hprt* mutants, the lack of sibling colonies suggests that this was not a likely source of error in the *Hprt* mutation assays.

Summary.

After both UVC and γ-irradiation, clonogenic survival was reduced in a dose-dependent manner but was consistently higher in *p53*-null cells. Following UVC-irradiation, a large dose-dependent increase in mutation frequency was observed but surprisingly, the frequency in *p53*-null cells was only two-fold higher than that in wild-type cells. In contrast, subsequent to γ-irradiation, a much smaller increase in mutation frequency was detected with no significant difference between wild-type and *p53*-null cells. Analysis of the mutagenic events leading to loss of HPRT activity after γ-irradiation revealed that at least 16 of the 22 clones analysed had undergone recombination events.

CHAPTER FOUR

DISCUSSION

4.1 Introduction.

The aims of this study were twofold. First to examine the effects of a *p53*-null environment on the induction of apoptosis in response to DNA-damage and to see how this translated to survival in the longer term. Second, to determine the role of p53 in the acquisition of mutations, both with and without prior treatment with DNA-damaging agents.

4.2 Methodological considerations.

One of the basic questions raised at the outset of this thesis was the nature of cells that escape apoptosis as a result of defects in the pathway coupling DNA damage to death. The techniques used in this thesis are able to accurately assess immediate death, in terms of apoptosis, and ultimate clonogenic survival. However, to draw quantitative conclusions linking numbers of cells that escape apoptosis to ultimate survivorship requires knowledge not only of levels of apoptosis but also of cellular proliferation and cell cycle arrest and how these vary over the time course of the clonogenic assay. Whilst it is possible to accurately measure these parameters at a single time point, an assessment over such a long time period is difficult.

Survival of cells at low density is known to be considerably poorer than at high density and thus survivorship in clonogenic assays, where cell density is low, might appear different from the survivorship of bulk cultures where cell density is higher (Freshney, 1983). Consequently, a quantitative assessment of the absolute number of mutant cells surviving DNA damage is difficult as this requires knowledge of both the mutation frequency, which was assessed in bulk cultures, and clonogenic survival.

Variations in cell density may also account for the observed disparity in the levels of apoptosis induced in otherwise identical experiments after both UVC and particularly γ-irradiation. This possibility was investigated in a series of experiments comparing levels of confluence at the time of irradiation with the induction of apoptosis. Data obtained from these experiments did suggest that cells irradiated at either very low or high levels of confluence might be more resistant to the induction of apoptosis. However, some variation was still observed between experiments even when cell density was controlled for, indicating other causative factors. Such a relationship might be explained in terms of the levels of cytokines and other metabolites to be found in the tissue culture medium at differing levels of confluence. Whilst tissue culture medium supplies a variety of growth factors, and in this case was supplemented with LIF, ES cells also produce their own growth and differentiation factors (Heath *et al.*, 1990). There is little data available on the effects of these cytokines on ES cell survival and proliferation at low cell density but the reduced proliferation of low density cultures suggests that these cytokines might be more relevant to ES cell survival and proliferation than those supplied artificially. In addition other metabolites that are able to pass through the cell membrane, for example low molecular weight intermediary metabolites, would also be present only at low levels in sparse cultures resulting in a steeper diffusion gradient than in dense cultures. Thus at very low confluence levels, due to the scarcity of cells, levels of relevant cytokines and intermediary metabolites would be low whilst at high confluence levels they would be elevated. This might be expected to affect the cellular sensitivity to apoptosis in several ways. First, in sparse cultures cellular proliferation might be expected to be reduced and there is evidence to suggest that some cell types are less susceptible to apoptosis when not actively cycling (Allan *et al.*, 1992; Evan *et al.*, 1992; Ling *et al.*, 1995; Olive *et al.*, 1996). Second, depletion of nutrients at higher confluence levels could also result in a reduction in the proportion of proliferating cells and a consequent increase in the observed resistance to apoptosis. In support of this theory, a study correlating cell cycle position with confluence levels in these same cells has indicated that the proportion of cells in S-

phase is lower in densely populated cultures (D. Connaughton, *personal communication*). Finally, a number of studies have indicated that a variety of growth factors can also act as survival factors and thus, at higher cell densities where levels of relevant cytokines might be elevated, apoptosis might be reduced (Collins *et al.*, 1992; Johnson *et al.*, 1993; Yonish-Rouach *et al.*, 1993; Gottlieb *et al.*, 1994; Canman *et al.*, 1995). The experiments described in this study only assayed the effects of confluence on apoptosis at a single time point and thus cannot exclude the possibility that the observed variations are the result of alterations in the kinetics of the induction of apoptosis rather than changes in sensitivity. However, clonogenic survival in ES cells has been found to be correlated with confluence levels at the time of UVC-irradiation (D. Connaughton, *personal communication*) and in other cell types, has been shown to vary through the cell cycle (Hall, 1994).

Whilst confluence levels at the time of irradiation may account for some of the variation in levels of apoptosis between experiments, there are clearly other causative factors involved. The exact nature of these factors remains unclear. Cells were derived from the same bulk freezes, experiments were set up in an identical manner and, as far as it was possible to determine, culture conditions identical between experiments. In most instances, culture medium constituents from the same batch were used in consecutive experiments and where this was not possible, components were batch tested before use. One possible source of variation that has not been eliminated is that batches of components were tested under normal culture conditions and the possibility remains that batches that sustain ES cell growth under these conditions may differ in their performance when cultures are placed under high stress. However, batches of components were large and consequently rarely changed suggesting that differences in the culture medium are not likely to have been the source of the observed variation between experiments.

Quantitative survival measurements require accurate dosimetry and whilst dosimetry is well established for γ-irradiation, no equivalent data was available for the UV spectrolinker. In addressing this issue, it became clear that although this apparatus is

frequently used in biological studies of the type presented here (Lu and Lane, 1993; Prost *et al.*, 1998) there are several problems associated with its use which required both recalibration of the apparatus and changes in experimental design. Even if the sensor, which was found to be faulty, were to be fully operational, the machine is not capable of accurately delivering doses in the range commonly used in these types of experiments. In addition, the lack of uniformity in light intensity in the irradiation chamber, and shadow effect of cell culture vessels would be likely to further reduce the dose accuracy. In many experiments where doses are not compared these errors would not significantly affect the overall conclusions drawn from the data but they would affect comparisons between data sets. The data clearly indicate the need to calibrate and test all UVC-irradiation equipment prior to use, even if new as was the apparatus used here.

4.3 The immediate response to DNA damage.

4.3.1 p53 and the apoptotic response to DNA-damaging agents.

In agreement with previous reports on both ES cells and other cell types, the data show that there is a rapid induction and nuclear localisation of p53 protein in wild type ES cells following exposure to UVC light (Maltzman and Czyzyk, 1984; Prost *et al.*, 1998) and that this induction appears to be associated with the initiation of apoptosis (Lu and Lane, 1993). A similar rapid induction of apoptosis was seen subsequent to treatment with the topoisomerase inhibitors, camptothecin and etoposide and has previously been observed in embryonic carcinoma (EC) cells, a cell type closely related to ES cells (Lutzker and Levine, 1996). As has formerly been shown in both ES cells and other cell types, the apoptotic response to UVC-irradiation was found to be reduced in *p53*-null cells and showed an intermediate response in heterozygote cell lines (Ziegler *et al.*, 1994; Sabapathy *et al.*, 1997). The p53-dependence of the response to etoposide was more complex, with significant levels of apoptosis being seen in *p53*-null cells only at higher doses. A similar partial dependence of the induction of apoptosis on p53 has previously been seen in

thymocytes following etoposide treatment (Clarke *et al.*, 1993). A statistically significant component of the *p53*-null cultures entered apoptosis indicating the presence of p53-independent apoptosis. Other studies on the effects of UVC-irradiation on *p53*-null ES cells have shown these cells do undergo apoptosis in response to this type of damage but that it is delayed relative to the kinetics of wild-type cells (D. Connaughton, *personal communication*). In addition, Aladjem *et al.* (1998) have shown that the apoptotic response to adriamycin in ES cells is p53-independent. Thus, taken together with published data, the studies presented here indicate that ES cells respond to DNA damaging agents with the induction of apoptosis by routes only partially p53-dependent. The general importance of this damage associated, p53-independent pathway is indicated by the fact that it has been observed in many other cell types (Strasser *et al.*, 1994; Malcomson *et al.*, 1995; Li *et al.*,1996; Aladjem *et al.*, 1998).

In contrast, the response to γ-irradiation was very different with little induction of apoptosis observed subsequent to this type of DNA damage. However, two distinct phases of p53 expression were detected. The function of the immediate p53 response to γ-irradiation is uncertain as mock-irradiated cells showed a similar induction suggesting that the response might be stress related. Mock UVC-irradiated controls also showed a transitory elevation in p53 expression. Induction of p53 in response to various stress stimuli, including hypoxia and heat-shock, has been reported by other authors (Sugano *et al.*, 1995; Renzing and Lane, 1995; Graeber *et al.*, 1994) and thus seems a likely explanation in this instance. This explanation concurs with the fact that the exposure of the cells to γ-irradiation, which involved substantially more disruptions of culture conditions than did UVC-irradiation, resulted in a higher proportion of positive staining cells among the handling-controls. A number of other studies have shown a rapid and transitory induction of p53 subsequent to ionising-radiation (Lu and Lane, 1993) but if this p53 response does occur in ES cells it would clearly be masked by the observed stress response and thus its presence can neither be confirmed or eliminated. A second, delayed phase of p53 induction, which peaked 24 hours subsequent to γ-irradiation, was observed in the

immunocytochemical analysis but was not detected in the Western analysis. The immunocytochemical method used here measures the proportion of cells showing upregulation of p53 expression, irrespective of the degree of induction, whereas Western analysis gives an estimate of the average cellular level of p53 protein and thus, these two methods measure different parameters. An explanation compatible with these differences would be that levels of induction, although widespread, are low. Alternatively, this second peak of expression might be associated with the initiation of apoptosis and differences between the two analyses could be attributed to the observed variability in the induction of apoptosis after this type of DNA-damage. A similar small increase in p53 expression was observed 18 hours after γ-irradiation by Aladjem *et al.* (1998) in another study on ES cells and confirms the importance of this finding. In addition, a biphasic apoptotic response to ionising radiation has been observed in both ES (Clutton *et al.*, 1996) and EC cells (Langley *et al.*, 1994), the timing of which closely follows the expression pattern of p53 seen here. It is possible that the induction of p53 detected here is associated with the induction of apoptosis but that the levels of apoptosis induced were outside the detection range of the assays used in this study. The absence of an immediate apoptotic response in ES cells is consistent with this cell type being highly resistant to ionising radiation but does not necessarily imply that these cells would respond to higher doses of radiation in the same manner. Studies on other cell types have shown that those that are resistant to radiation, in terms of apoptosis, will often display delayed apoptotic death at low doses but undergo a rapid wave of apoptosis at higher doses (reviewed by Held, 1997).

The absence of an immediate wave of apoptosis following γ-irradiation is surprising considering the kinetics of DNA damage anticipated. γ-irradiation damages DNA directly with single strand breaks (ssb), base and sugar damage and a smaller number of double strand breaks being effected within milliseconds of the ionisation events. The most commonly formed lesions subsequent to UVC exposure however, are cyclobutane pyrimidine dimers and 6-4 photoproducts, which might not be expected to immediately signal stabilisation of p53 and thence apoptosis, and which are

converted to ssbs in a second set of reactions during NER in the damaged cells. Direct measurements of the kinetics of repair subsequent to γ-irradiation are not available for ES cells, but studies in other cell types suggest that the majority of lesions are repaired rapidly, usually well within four hours (see Powell and McMillan, 1990). In both ES and EC cells the majority of lesions caused by UVC-irradiation are repaired within 24 hours but repair is not complete until 48 hours after irradiation (Rasko *et al.*, 1993; Prost *et al.*, 1998). Similarly, although etoposide and camptothecin induce strand breaks, repair of these lesions is delayed as the cleavable complex formed by the binding of the topoisomerase inhibitors to topoisomerases is bound to the DNA and thus blocks repair. The apparently contradictory nature of these observations highlights certain questions. First, what is the trigger for p53-dependent apoptosis in UVC damaged cells? Second, what is the trigger in the absence of p53 and is it as efficient? Last, why do ES cells fail to enter apoptosis subsequent to γ-irradiation?

4.3.3 Cell cycle checkpoints in embryonic stem cells.

In agreement with previous reports on ES cells, a G_2/M arrest was observed following ionising radiation (Clutton *et al.*, 1996). The arrest occurred in both wild-type and *p53*-null cells and was maximal six hours after irradiation, persisting for more than 24 hours. In their study on ES cells, Aladjem *et al.* (1998) were unable to detect this arrest 18 hours after γ-irradiation although, in the data presented here, even 24 hours after irradiation, a highly significant increase in the proportion of G_2/M cells was observed in irradiated cultures. The reason for this discrepancy is not clear, although Aladjem *et al.* do state that "no reproducible" G_1 or G_2/M arrest was seen, suggesting that they may have observed an arrest in at least one experiment. An S phase arrest has been observed in ES cells following UVC-irradiation, but this was also p53-independent (Prost *et al.*, 1998). In fact, despite several studies on the effects of DNA-damaging agents on wild-type and *p53*-null ES cells, there are no reports in the literature demonstrating p53-dependent cell cycle checkpoints and it has been suggested that this p53-dependent response to DNA-damage is absent in this cell type (Aladjem *et al.*, 1998). Relative to other cell types, undifferentiated ES

and EC cells express high levels of p53 protein in the absence of injury and yet are able to proliferate rapidly (Rogel *et al.*, 1985; Li *et al.*, 1996; MacCallum *et al.*, 1996; Sabapathy *et al.*, 1997). Several authors have suggested that this is possible because p53 is functionally inactive, at least in terms of transcriptional activation, in these cells (Lutzker and Levine, 1996; Aladjem *et al.*, 1998), but evidence for this is contradictory (Sabapathy *et al.*, 1997). However, induction of p53-dependent apoptosis in EC cells by etoposide did result in upregulation of p53 and transcriptional activation of p53 targets (Lutzker and Levine, 1996). Taken together, these results suggest that ES cells are able to tolerate high, basal levels of wild-type p53 and that it is only when p53 is upregulated in response to DNA damage that induction of either cell cycle arrest or apoptosis follows. In the differentiated derivatives of ES cells these high levels of expression are reduced and a proportion of the p53 protein undergoes a conformational change to the form inactive for transcriptional transactivation (Rogel *et al.*, 1985; Sabapathy *et al.*, 1997). In addition, differentiation of ES cells restores the cell cycle arrest response to *n*-phosphonacetyl-L-aspartate (PALA) treatment (Aladjem *et al.*, 1998).

4.4 p53 and cell survival.

Clonogenic survival was also influenced by *p53* genotype. After both γ- and UVC-irradiation the survival of *p53*-null cells, compared to wild-type, was enhanced in a dose dependent manner. As predicted, a log linear response was observed following both UVC- and γ-irradiation, at least down to the level of 1% survival. In a previous study examining clonogenic survival in ES cells after X-irradiation, Clutton *et al.* (1996) found similar levels of survival to those observed in this study after low doses of radiation (3.5 Gy and less) but significantly lower survival after high doses (10 Gy). The reason for this discrepancy is not entirely clear as the cell lines used were closely related to those used in this study. The clonogenic assay used by Clutton *et al.* was substantially different from the one used in this study, the main differences being that trypsinised cells were passed through a 23 gauge needle to remove cell clumps before being irradiated in suspension and that cells were cultured in medium

supplemented with low melting point agarose. One possible explanation for the observed differences in survival in the two studies might be that Clutton *et al.* irradiated their cells in suspension, rather than as monolayers in flasks, which might have adversely affected cell survival. However, Clutton *et al.* investigated this possibility and found no differences in survival between the two irradiation protocols (E. Wright, *personal communication*). Although the clonogenic assay used here did show linearity over the range of plating densities examined it is not possible to examine linearity at the higher densities required where high doses of DNA-damaging agents are used. Thus it is possible that the differences in clonogenic survival observed in the two studies are the result of a breakdown in linearity at high cell density.

The absence of a wave of apoptosis immediately after γ-irradiation would be consistent with the interpretation that either death is dependent on secondary DNA breakage, perhaps effected during post-irradiation replication events or that the cells do not die, but fail to proliferate. There is evidence in the literature for both of these processes playing an important role in the response to ionising radiation. It has long been known that lethally irradiated cells may undergo several cell divisions before they lose their proliferative capacity (Thompson and Suit, 1969) and Seymour *et al.* (1986) observed significantly decreased cloning efficiency as late as 30 cell divisions post-irradiation. Similar results have been obtained with ES cells subsequent to X-irradiation and α-particles (Clutton *et al.*, 1996). In a later series of experiments, Seymour and Mothersill (1992) found that this reduction in cloning efficiency was the result of the production of sterile cells, many of which are abnormal in appearance and which fail to form viable colonies on replating. Studies on cell cycle progression of G_0-G_1 synchronised wild-type and *p53*-null fibroblasts after γ-irradiation have shown that, of those cells which escape the initial G_0-G_1 phase, many undergo a permanent arrest in subsequent phases but that the proportion of cells arresting is substantially lower in *p53*-null cells than in wild-type cells (Linke *et al.*, 1997). In addition, it has been demonstrated that apoptosis can occur in irradiated

cells post-mitotically (Radford and Murphy, 1994; Tauchi *et al.*, 1994; Yanagihara *et al.*, 1995; Vidair *et al.*, 1996)

These results clearly demonstrate that clonogenic survival is increased in *p53*-null cells but do not permit conclusions on the basis of this effect. It is tempting to conclude, from the UVC data, that it relates to early apoptosis but the γ survival data do not reveal sufficient information on the p53-dependence of late death, if indeed the reduction in survivorship is the result of death, for any conclusions to be drawn. However, it is clear that the magnitude of the immediate apoptotic response is a very poor indicator of the ultimate clonogenic survival, at least in response to γ-irradiation.

4.5 p53 and the mutation frequency.

The majority of studies to date investigating the effect of p53 on the spontaneous mutation frequency of normal, murine cells have indicated that *p53*-null cells do not bear an increased mutation load (Nishino *et al.*, 1995; Sands *et al.*, 1995; Clarke *et al.*, 1997) and the data obtained in this study on the spontaneous mutation frequency of the *Hprt* gene is in agreement with these reports. These findings are further supported by interbreeding experiments between *Apc* mutated (Min) and *p53*-null mice which show no evidence for an increase in the spontaneous rate of adenoma formation in *Apc* +/-, *p53* -/- mice compared with *Apc* +/-, *p53* +/+ animals. As adenoma formation is associated with the loss of the remaining wild-type *Apc* allele, this suggests that the absence of p53 does not increase the spontaneous mutation frequency in these mice (Clarke *et al.*, 1995). However, p53 status clearly does influence spontaneous tumorigenesis in many other cell types, as demonstrated by the phenotype of the *p53*-null mice (Donehower *et al.*, 1992; Jacks *et al.*, 1994; Purdie *et al.*, 1994) and by several different *p53* intercrosses (Donehower *et al.*, 1995; Clarke *et al.*, 1995). It might be hypothesised from these results that the effects of a *p53*-null background on genomic instability and the acquisition of mutations at spontaneous levels of DNA damage are dependent upon cell type but as it is not known to what

level the tissues examined were subjected to naturally occurring carcinogens it is difficult to draw conclusions.

It is implicit from the strong association between DNA damage and p53 induction that changes in *p53* status may have significant effects on the mutation frequency only following exposure to DNA damage. Surprisingly, an analysis of mutation at the *Hprt* locus in cells surviving either γ– or UVC-irradiation showed that mutation frequency within surviving clones is largely uninfluenced by *p53* status. We did observe a small (two fold) but statistically significant increase following UVC irradiation. The simplest explanation for these results is that efficient NER has a more stringent requirement for functional p53 than does repair of γ-irradiation induced lesions and, as previously discussed, there is evidence in support of this hypothesis. This explanation is not, however, supported by experiments using *p53*-null Big Blue embryonic fibroblasts treated with the UV mimetic 4-nitroquinoline 1-oxide (4NQO), which show no increase in mutation frequency (Sands *et al.*, 1995). One explanation for this discrepancy would be that again, the requirement for p53 may vary between cell types. A potential source of error in the type of assay used in this study is that one or several mutant clones might acquire a growth advantage resulting in an overestimation of the mutation frequency. However, it seems unlikely that the data presented here are affected by such errors as an analysis of the clonal origins of eight irradiated, 6-TG resistant, *p53*-null clones revealed no sibling colonies and, although it was not possible to examine the clonal origins of all *Hprt* mutants, estimates of mutation frequency were consistent between duplicated experiments. The assay used in this study measures the proportion of surviving cells which bear mutations but does not take into account any increases in the number of cells that survive. Thus, although loss of p53 does not result in an increased mutation frequency following γ-irradiation, the number of mutants surviving irradiation is greater as a consequence of the increased survival of *p53*-null cells. Similarly, subsequent to UVC-irradiation, survival of mutant cells is increased in the absence of p53 due to both the increased mutation frequency and increased survival.

4.6 p53 and chromosomal instability.

Previous studies of embryonic fibroblasts derived from *p53*-null mice and many *p53*-null tumour cell lines have shown these cells to have unstable karyotypes when cultured *in vitro* (Livingstone *et al.*, 1992; Tsukada *et al.*, 1993; Meling *et al.*, 1993; Carder *et al.*, 1993; Purdie *et al.*, 1994; Donehower *et al.*, 1995). Although the ES cell lines examined did possess some chromosomal abnormalities, they showed no tendency to acquire further abnormalities over a ten week period of culture. This is in contrast to *p53*-null embryonic fibroblasts which over a similar period of growth have been shown to display an increasing tendency towards aneuploidy (Livingstone *et al.*, 1992; Harvey *et al.*, 1993; Tsukada *et al.*, 1993).

Trisomy of targeted chromosomes has not been observed in other doubly targeted cell lines derived using the same method (Mortensen *et al.*, 1992) but appeared here in three out of four *p53*-null cell lines, derived in two separate experiments and representing at least three unrelated clones. Whilst other possibilities exist, the most likely explanation is that chromosome 11 has an inherent tendency towards trisomy and this would be accentuated by the high G418 selection. This hypothesis is supported by our own observations and several reports of spontaneous occurrences of trisomy 11 in both ES and EC cells (McBurney, 1976; McBurney and Rogers, 1982; Robertson and Bradley, 1986; Crolla *et al.*, 1990).

It is possible some of the results obtained here arise as a consequence of chromosome 11 trisomy. However, we have observed normal growth and differentiation in these ES cells and hence consider that compared to the majority of *p53*-null cell lines examined, they represent a relatively karyotypically normal cell line.

4.7 Conclusions.

This study has established a limited role for p53 in determining mutation within clones surviving UVC-irradiation. However, the results described here derive from a direct comparison of mutation amongst clonogenic cells. To understand the true

consequences of p53 deficiency, the p53 dependency of clonogenicity must also be considered. Thus, following γ-irradiation, although the proportion of mutant clones is unaffected by *p53* status, there is a dose-dependent increase in the number of *Hprt* mutant clones as a direct consequence of the increased clonogenicity of *p53*-null cells. Similarly, following UVC irradiation, there is an increase in the number of mutant clones, both as a result of the increased mutation frequency and as a consequence of increased clonogenicity. Results similar to those presented here have been obtained from an analysis of clonogenic IL7-dependent pre-B cells, which has shown that the number of clones mutant at the *Hprt* locus is p53 dependent at relatively low doses of γ-irradiation (Griffiths *et al.*, 1997). As with ES cells, this p53-dependent difference largely arises as a consequence of increased clonality. However, our data contrast with a parallel study (Clarke *et al.*, 1997) which scored the mutation frequency at the *Dlb1* locus in clonogenic intestinal cells following γ-irradiation. This study indicated a role for p53 in preventing mutation, but only at high doses (6 Gy). One hypothesis which would explain such cell lineage differences would be that cell types differ in their reliance upon p53-dependent and -independent mechanisms and also in their relative use of these mechanisms. Hence, cells which survive genotoxic insult may be regarded as the end product of a complex equilibrium of processes, some of which are dependent on and others independent of p53, and that the relative importance of these processes varies between cell type and in response to different forms of DNA damage. Similarly, for a given cell type loss of p53-dependent mechanisms may only be significant at specific levels of a given type of DNA damage. In support of this hypothesis, we are able to demonstrate the existence of both p53-dependent and -independent pathways in ES cells. This hypothesis might also explain the difference between the UVC experiments presented here and those of Sands *et al.* (1995) and also between UVC and γ-irradiation in this study, as p53-dependent mechanisms may not have been limiting at the doses examined in these experiments.

4.8 Future research directions.

The precise nature of the relationship between apoptosis and clonogenicity has received relatively little attention. Various studies have attempted to examine this relationship by looking at the induction of apoptosis in genetically similar cell lines that differ in their clonogenic sensitivity. In many cases, a correlation between these two variables has been found (Story *et al.*, 1994; Han *et al.*, 1995; Russell *et al.*, 1995) but this has not always been the case (Radford, 1994; Radford and Murphy, 1994; Xia *et al.*, 1995). An alternative approach to this question has been to assess the effects on clonogenic survival of treatments that modify the apoptotic response, for example calcium chelation, caffeine and oxygen levels. Again results have been conflicting and it appears that the type of modifying agent used is important (Bernhard *et al.*, 1996; Hopcia *et al.*, 1996; Voehringer *et al.*, 1997). For example, calcium chelating agents might alter only the relatively late stage DNA cleavage and merely repress the apoptotic death of cells that are nonetheless committed to die. Another question that remains to be answered satisfactorily is whether or not apoptosis can account for all of the cell loss in clonogenicity. Where studies have assessed apoptosis only in the short term, the answer seems to be no (Dewey *et al.*, 1995; Hopcia *et al.*, 1996) but some studies have suggested that if adequate time is allowed for the cells to undergo apoptosis then it can account for all cell deaths (Olive *et al.*, 1996). All of these studies highlight the importance of assaying not only immediate apoptosis but also delayed apoptosis. With the techniques commonly used today, this is not usually possible. Most assays measure apoptosis only at a single time point or up to the time of the assay. Also, where apoptosis is delayed cells may divide several times before dying. All of these problems could be circumvented with the use of time lapse microscopy.

A number of studies, including an analysis of the p53-dependence of the apoptotic response to UVC-irradiation in ES cells, have suggested that loss of p53 does not abrogate the apoptotic response to DNA-damage but merely delays it (Radford, 1994; Xia *et al.*, 1995; D. Connaughton, *personal communication*). A comparison of the early apoptotic response to UVC-irradiation and the resulting clonogenic survival

curve in *p53*-null ES cells observed in this investigation clearly indicates the existence of p53-independent pathways to either cell death or the loss of proliferative potential. Whether this loss of clonogenic potential results from a delayed, p53-independent wave of apoptosis could be addressed by the use of time lapse microscopy. In addition, there are several unanswered questions about the relationship between apoptosis and clonogenic survival that could also be addressed in such a study. For example; what proportion of clonogenic death can be accounted for by apoptosis (immediate or delayed) and what role does p53 play in this apoptosis, what is the role of differentiation in clonogenic loss and to what extent does the type of DNA damaging agent used affect the relative importance of these processes.

Another question that remains to be answered satisfactorily is whether the sensitivity to DNA-damaging agents differs between quiescent and proliferating cells. The data presented in this study on the effects of confluence on the induction of apoptosis suggest that this may indeed be the case, but a more rigorous investigation is clearly required. An analysis of apoptotic sensitivity utilising cell cycle stage as a predictor might produce clearer results. In addition, a careful investigation of any changes in the kinetics of the induction of apoptosis and the long term consequences for clonogenic survival would also be required.

In the analyses of p53 induction following γ-irradiation presented here, a biphasic response was seen in the Western analyses. The second peak of p53 expression was not observed in the immunocytochemical analyses but it is not clear whether this was due to differences in the parameters that these two types of assays measure or because this second phase of expression is associated with the induction of apoptosis, a response that is itself variable after this type of DNA-damaging agent. Concurrent analyses of the induction of both p53 and apoptosis would address this problem.

At a more general level, why is it that some cell types are more sensitive to the induction of apoptosis following ionising radiation than others? Studies in most

normal tissues have shown a rapid induction of apoptosis within a few hours of irradiation in both haematopoietic and non-haematopoietic cells. In tumour derived cells, results are less consistent and many cell lines show either an increased resistance to apoptosis or a delay in its induction (reviewed by Held, 1997). The data presented in this study show very little induction of apoptosis (following γ-irradiation) in ES cells: a cell type which is both normal and rapidly proliferating and thus might be expected to be very sensitive to the induction of apoptosis. It is possible that the observed variation in tumour cell lines is the result of the acquisition of genetic lesions but this seems an unlikely explanation in ES cells. Thus, the data suggest that ES cells are in some way different from most normal cell types and an analysis of the molecular basis of this difference would be an appropriate area for future research.

Bibliography.

Agarwal, M.L., Agarwal, A., Taylor, W.R., and Stark, G.R. (1995) p53 controls both the G_2/M and the G_1 cell cycle checkpoints and mediates reversible growth arrest in human fibroblasts. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **92**, 8493-8497.

Aladjem, M.I., Spike, B.T., Rodewald, L.W., Hope, T.J., Klemm, M., Jaenisch, R., and Wahl, G.M. (1998) ES cells do not activate p53-dependent stress responses and undergo p53-independent apoptosis in response to DNA damage. *Current Biology* **8**, 145-155.

Allan, D.J. (1992) Radiation induced apoptosis - its role in a madcat (mitosis-apoptosis-differentiation-calcium toxicity) scheme of cytotoxicity mechanisms. *International Journal Of Radiation Biology* **62**, 145-152.

Allen, T.D. (1987) Ultrastructural aspects of cell death. *In: Perspectives on Mammalian Cell Death Potten, C.S. (ed.)* Oxford University Press, Oxford. 36-65.

Alonigrinstein, R., Schwartz, D., and Rotter, V. (1995) Accumulation of wild-type p53 protein upon gamma-irradiation induces a G_2 arrest-dependent immunoglobulin kappa light-chain gene expression. *Embo Journal* **14**, 1392-1401.

Armstrong, J.F., Kaufman, M.H., Harrison, D.J., and Clarke, A.R. (1995) High-frequency developmental abnormalities in p53-deficient mice. *Current Biology* **5**, 931-936.

Avantaggiati, M.L., Ogryzko, V., Gardner, K., Giordano, A., Levine, A.S., and Kelly, K. (1997) Recruitment of p300/CBP in p53-dependent signal pathways. *Cell* **89**, 1175-1184.

Bakalkin, G., Yakovleva, T., Selivanova, G., Magnusson, K.P., Szekely, L., Kiseleva, E., Klein, G., Terenius, L., and Wiman, K.G. (1994) p53 binds single-stranded DNA ends and catalyzes DNA renaturation and strand transfer. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **91**, 413-417.

Banerjee, D., Lenz, H.J., Schnieders, B., Manno, D.J., Ju, J.F., Spears, C.P., Hochhauser, D., Danenberg, K., Denenberg, P., and Bertino, J.R. (1995) Transfection of wild-type but not mutant p53 induces early monocytic differentiation in HL-60 cells and increases their sensitivity to stress. *Cell Growth & Differentiation* **6**, 1405-1413.

Barak, Y., Juven, T., Haffner, R., and Oren, M. (1993) MDM2 expression is induced by wild type p53 activity. *Embo Journal* **12**, 461-468.

Bargonetti, J., Friedman, P.N., Kern, S.E., Vogelstein, B., and Prives, C. (1991) Wild-type but not mutant p53 immunopurified proteins bind to sequences adjacent to the SV40 origin of replication. *Cell* **65**, 1083-1091.

Bayle, J.H., Elenbaas, B., and Levine, A.J. (1995) The carboxyl-terminal domain of the p53 protein regulates sequence- specific DNA binding through its nonspecific nucleic acid binding activity. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **92**, 5729-5733.

Bernhard, E.J., Muschel, R.J., Bakanauskas, V.J., and McKenna, W.G. (1996) Reducing the radiation-induced G_2 delay causes hela cells to undergo apoptosis instead of mitotic death. *International Journal Of Radiation Biology* **69**, 575-584.

Bischoff, F.Z., Yim, S.O., Pathak, S., Grant, G., Siciliano, M.J., Giovanella, B.C., Strong, L.C., and Tainsky, M.A. (1990) Spontaneous abnormalities in normal fibroblasts from patients with Li-Fraumeni cancer syndrome - aneuploidy and immortalization. *Cancer Research* **50**, 7979-7984.

Blunt, T., Finnie, N.J., Taccioli, G.E., Smith, G.C.M., Demengeot, J., Gottlieb, T.M., Mizuta, R., Varghese, A.J., Alt, F.W., Jeggo, P.A., and Jackson, S.P. (1995) Defective DNA-dependent protein kinase activity is linked to V(D)J recombination and DNA repair defects associated with the murine SCID mutation. *Cell* **80**, 813-823.

Boldin, M.P., Goncharov, T.M., Goltsev, Y.V., and Wallach, D. (1996) Involvement of MACH, a novel MORT1/FADD-interacting protease, in FAS/APO-1-induced and TNF receptor induced cell death. *Cell* **85**, 803-815.

Bouffler, S.D., Kemp, C.J., Balmain, A., and Cox, R. (1995) Spontaneous and ionizing radiation induced chromosomal abnormalities in p53-deficient mice. *Cancer Research* **55**, 3883-3889.

Boulikas, T. (1996) Xeroderma pigmentosum and molecular cloning of DNA repair genes. *Anticancer Research* **16**, 693-708.

Boyd, J.M., Gallo, G.J., Elangovan, B., Houghton, A.B., Malstrom, S., Avery, B.J., Ebb, R.G., Subramanian, T., Chittenden, T., Lutz, R.J., and Chinnadurai, G. (1995) BIK, a novel death-inducing protein shares a distinct sequence motif with BCL-2 family proteins and interacts with viral and cellular survival-promoting proteins. *Oncogene* **11**, 1921-1928.

Brain, R. and Jenkins, J.R. (1994) Human p53 directs DNA strand reassociation and is photolabeled by 8- azido ATP. *Oncogene* **9**, 1775-1780.

Brenner, L., Munozantonia, T., Vellucci, V.F., Zhou, Z.L., and Reiss, M. (1993) Wild-type p53 tumor suppressor gene restores differentiation of human squamous carcinoma cells but not the response to transforming growth factor-beta. *Cell Growth & Differentiation* **4**, 993-1004.

Brugarolas, J., Chandrasekaran, C., Gordon, J.I., Beach, D., Jacks, T., and Hannon, G.J. (1995) Radiation induced cell cycle arrest compromised by p21 deficiency. *Nature* **377**, 552-557.

Buckbinder, L., Talbott, R., Velascomiguel, S., Takenaka, I., Faha, B., Seizinger, B.R., and Kley, N. (1995) Induction of the growth inhibitor IGF-binding protein-3 by p53. *Nature* **377**, 646-649.

Caelles, C., Helmberg, A., and Karin, M. (1994) p53-dependent apoptosis in the absence of transcriptional activation of p53 target genes. *Nature* **370**, 220-223.

Canman, C.E., Gilmer, T.M., Coutts, S.B., and Kastan, M.B. (1995) Growth factor modulation of p53-mediated growth arrest versus apoptosis. *Genes & Development* **9**, 600-611.

Carder, P., Wyllie, A.H., Purdie, C.A., Morris, R.G., White, S., Piris, J., and Bird, C.C. (1993) Stabilized p53 facilitates aneuploid clonal divergence in colorectal cancer. *Oncogene* **8**, 1397-1401.

Chen, J.D., Marechal, V., and Levine, A.J. (1993a) Mapping of the p53 and MDM-2 interaction domains. *Molecular And Cellular Biology* **13**, 4107-4114.

Chen, X.B., Farmer, G., Zhu, H., Prywes, R., and Prives, C. (1993b) Cooperative DNA-binding of p53 with TFIID (TBP) - a possible mechanism for transcriptional activation. *Genes & Development* **7**, 1837-1849.

Cheng, E.H.Y., Levine, B., Boise, L.H., Thompson, C.B., and Hardwick, J.M. (1996) BAX-independent inhibition of apoptosis by BCL-X(L). *Nature* **379**, 554-556.

Chinnaiyan, A.M., Orth, K., O'Rourke, K., Duan, H.J., Poirier, G.G., and Dixit, V.M. (1996) Molecular ordering of the cell death pathway - BCL-2 and BCL-X(L) function upstream of the CED-3-like apoptotic proteases. *Journal Of Biological Chemistry* **271**, 4573-4576.

Chinnaiyan, A.M., Chaudhary, D., O'Rourke, K., Koonin, E.V., and Dixit, V.M. (1997a) Role of CED-4 in the activation of CED-3. *Nature* **388**, 728-729.

Chinnaiyan, A.M., O'Rourke, K., Lane, B.R., and Dixit, V.M. (1997b) Interaction of CED-4 with CED-3 and CED-9: a molecular framework for cell death. *Science* **275**, 1122-1126.

Chiou, S.K., Rao, L., and White, E. (1994a) BCL-2 blocks p53-dependent apoptosis (vol 14, pg 2561, 1994). *Molecular And Cellular Biology* **14**, 4333

Cho, Y.J., Gorina, S., Jeffrey, P.D., and Pavletich, N.P. (1994) Crystal structure of a p53 tumor suppressor DNA complex -understanding tumorigenic mutations. *Science* **265**, 346-355.

Chow, S.C., Weis, M., Kass, G.E.N., Holmstrom, T.H., Eriksson, J.E., and Orrenius, S. (1995) Involvement of multiple proteases during FAS-mediated apoptosis in T lymphocytes. *Febs Letters* **364**, 134-138.

Chowdary, D.R., Dermody, J.J., Jha, K.K., and Ozer, H.L. (1994) Accumulation of p53 in a mutant cell line defective in the ubiquitin pathway. *Molecular And Cellular Biology* **14**, 1997-2003.

Chu, G. and Chang, E. (1988) Xeroderma pigmentosum group E cells lack a nuclear factor that binds to damaged DNA. *Science* **242**, 564-567.

Chu, G. and Chang, E. (1990) Cisplatin-resistant cells express increased levels of a factor that recognizes damaged DNA. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **87**, 3324-3327.

Clarke, A.R., Purdie, C.A., Harrison, D.J., Morris, R.G., Bird, C.C., Hooper, M.L., and Wyllie, A.H. (1993) Thymocyte apoptosis induced by p53-dependent and independent pathways. *Nature* **362**, 849-852.

Clarke, A.R., Gledhill, S., Hooper, M.L., Bird, C.C., and Wyllie, A.H. (1994) p53 dependence or early apoptotic and proliferative responses within the mouse intestinal epithelium following gamma irradiation. *Oncogene* **9**, 1767-1773.

Clarke, A.R., Cummings, M.C., and Harrison, D.J. (1995) Interaction between murine germline mutations in *p53* and *Apc* predisposes to pancreatic neoplasia but not to increased intestinal malignancy. *Oncogene* **11**, 1913-1920.

Clarke, A.R., Howard, L.A., Harrison, D.J., and Winton, D.J. (1997) p53, mutation frequency and apoptosis in the murine small intestine. *Oncogene* **14**, 2015-2018.

Clutton, S.M., Townsend, K.M.S., Goodhead, D.T., Ansell, J.D., and Wright, E.G. (1996a) Differentiation and delayed cell death in embryonal stem cells exposed to low doses of ionizing radiation. *Cell Death And Differentiation* **3**, 141-148.

Collins, M.K.L., Marvel, J., Malde, P., and Lopezrivas, A. (1992) Interleukin-3 protects murine bone marrow cells from apoptosis induced by DNA damaging agents. *Journal Of Experimental Medicine* **176**, 1043-1051.

Crolla, J.A., Brown, D. and Whittingham, D.G. (1990) Spontaneous induction of an homologous Robertsonian translocation, Rb(11.11) in a murine embryonic stem cell line. *Genetic Research* **55**, 107-110.

Cross, S.M., Sanchez, C.A., Morgan, C.A., Schimke, M.K., Ramel, S., Idzerda, R.L., Raskind, W.H., and Reid, B.J. (1995) A p53-dependent mouse spindle checkpoint. *Science* **267**, 1353-1356.

Darmon, A.J., Nicholson, D.W., and Bleackley, R.C. (1995) Activation of the apoptotic protease CPP32 by cytotoxic T-cell-derived granzyme-B. *Nature* **377**, 446-448.

Debbas, M. and White, E. (1993) Wild-type p53 mediates apoptosis by E1A, which is inhibited by E1B. *Genes & Development* **7**, 546-554.

Demers, G.W., Foster, S.A., Halbert, C.L., and Galloway, D.A. (1994) Growth arrest by induction of p53 in DNA damaged keratinocytes is bypassed by human papillomavirus-16 E7. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **91**, 4382-4386.

Deng, C.X., Zhang, P.M., Harper, J.W., Elledge, S.J., and Leder, P. (1995) Mice lacking *p21(Cip1/Waf1)* undergo normal development, but are defective in G_1 checkpoint control. *Cell* **82**, 675-684.

Dewey, W.C., Ling, C.C., and Meyn, R.E. (1995) Radiation-induced apoptosis - relevance to radiotherapy. *International Journal Of Radiation Oncology Biology Physics* **33**, 781-796.

Dileonardo, A., Linke, S.P., Clarkin, K., and Wahl, G.M. (1994) DNA-damage triggers a prolonged p53-dependent G_1 arrest and long- term induction of CIP1 in normal human fibroblasts. *Genes & Development* **8**, 2540-2551.

Donehower, L.A., Harvey, M., Slagle, B.L., McArthur, M.J., Montgomery, C.A., Butel, J.S., and Bradley, A. (1992) Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumors. *Nature* **356**, 215-221.

Donehower, L.A., Godley, L.A., Aldaz, C.M., Pyle, R., Shi, Y.P., Pinkel, D., Gray, T., Bradley, A., Medina, D., and Varmus, H.E. (1995) Deficiency of *p53* accelerates mammary tumorigenesis in *Wnt-1* transgenic mice and promotes chromosomal instability. *Genes & Development* **9**, 882-895.

Dost, B. (1998) Analysis of the role of caspase 2/ICH-1 in apoptosis. *PhD thesis, University of Edinburgh.*

Duan, H.J., Chinnaiyan, A.M., Hudson, P.L., Wing, J.P., He, W.W., and Dixit, V.M. (1996) ICE-LAP3, a novel mammalian homolog of the *Caenorhabditis-elegans* cell-death protein CED-3 is activated during FAS and tumor necrosis factor-induced apoptosis. *Journal Of Biological Chemistry* **271**, 1621-1625.

Dulic, V., Kaufmann, W.K., Wilson, S.J., Tlsty, T.D., Lees, E., Harper, J.W., Elledge, S.J., and Reed, S.I. (1994) p53-dependent inhibition of cyclin-dependent kinase activities in human fibroblasts during radiation induced G_1 arrest. *Cell* **76**, 1013-1023.

Dutta, A., Ruppert, J.M., Aster, J.C., and Winchester, E. (1993) Inhibition of DNA replication factor RPA by p53. *Nature* **365**, 79-82.

Ehinger, M., Nilsson, E., Persson, A.M., Olsson, I., and Gullberg, U. (1995) Involvement of the tumor suppressor gene *p53* in tumor necrosis factor induced differentiation of the leukemic cell line K562. *Cell Growth & Differentiation* **6**, 9-17.

Eizenberg, O., Faberelman, A., Gottlieb, E., Oren, M., Rotter, V., and Schwartz, M. (1995) Direct involvement of p53 in programmed cell death of oligodendrocytes. *Embo Journal* **14**, 1136-1144.

Ekert, P.G. and Vaux, D.L. (1997) Apoptosis and the immune system. *British Medical Bulletin* **53** (No.3) 591-603.

El-Deiry, W.S., Kern, S.E., Pietenpol, J.A., Kinzler, K.W., and Vogelstein, B. (1992) Definition of a consensus binding site for p53. *Nature Genetics* **1**, 45-49.

Enari, M., Hug, H., and Nagata, S. (1995) Involvement of an ICE-like protease in FAS-mediated apoptosis. *Nature* **375**, 78-81.

Evan, G.I., Wyllie, A.H., Gilbert, C.S., Littlewood, T.D., Land, H., Brooks, M., Waters, C.M., Penn, L.Z., and Hancock, D.C. (1992) Induction of apoptosis in fibroblasts by C-MYC protein. *Cell* **69**, 119-128.

Fantes, J.A., Bickmore, W.A., Fletcher, J.M., Ballesta, F., Hanson, I.M., and Van Heyningen, V. (1992) Submicroscopic deletions at the *WAGR* locus, revealed by nonradioactive insitu hybridization. *American Journal Of Human Genetics* **51**, 1286-1294.

Farmer, G., Bargonetti, J., Zhu, H., Friedman, P., Prywes, R., and Prives, C. (1992) Wild-type p53 activates transcription *in vitro*. *Nature* **358**, 83-86.

Feinstein, E., Gale, R.P., Reed, J., and Canaani, E. (1992) Expression of the normal p53 gene induces differentiation of K562 cells. *Oncogene* **7**, 1853-1857.

Fernandes-Alnemri, T., Armstrong, R.C., Krebs, J., Srinivasula, S.M., Wang, L., Bullrich, F., Fritz, L.C., Trapani, J.A., Tomaselli, K.J., Litwack, G., and Alnemri, E.S. (1996) *In vitro* activation of CPP32 and MCH3 by MCH4, a novel human apoptotic cysteine protease containing 2 FADD-like domains. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **93**, 7464-7469.

Ford, J.M. and Hanawalt, P.C. (1995) Li-Fraumeni syndrome fibroblasts homozygous for p53 mutations are deficient in global DNA repair but exhibit normal transcription- coupled repair and enhanced uv resistance. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **92**, 8876-8880.

Frankenberg, D., Frankenberg-Schwager, M., Blocher, D., and Harbich, R. (1981) Evidence for DNA double strand breaks as the critical lesions in yeast cells irradiated with sparsely or densely ionizing radiation under oxic or anoxic conditions. *Radiation Research* **88**, 524-532.

Frankenberg-Schwager, M., Frankenberg, D., Blocher, D., Harbich, R., and Adamczyk, C. (1982) Irreparable DNA double strand breaks induced in eukaryotic cells by sparsely or densely ionizing radiation and their importance for cell killing. *Mutation Research* **96**, 132-133.

Freshney, R.I. (1983) Culture of animal cells: A manual of basic technique. Alan R. Liss, Inc., New York.

Friedman, P.N., Chen, X.B., Bargonetti, J., and Prives, C. (1993) The p53 protein is an unusually shaped tetramer that binds directly to DNA. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **90**, 3319-3323.

Fritsche, M., Haessler, C., and Brandner, G. (1993) Induction of nuclear accumulation of the tumor suppressor protein p53 by DNA damaging agents. *Oncogene* **8**, 307-318.

Fuchs, B., O'Connor, D., Fallis, L., Scheidtmann, K.H., and Lu, X. (1995) p53 phosphorylation mutants retain transcription activity. *Oncogene* **10**, 789-793.

Fukasawa, K., Wiener, F., Van de Woude, G.F., and Mai, S.B. (1997) Genomic instability and apoptosis are frequent in p53 deficient young mice. *Oncogene* **15**, 1295-1302.

Funk, W.D., Pak, D.T., Karas, R.H., Wright, W.E., and Shay, J.W. (1992) A transcriptionally active DNA binding site for human p53 protein complexes. *Molecular And Cellular Biology* **12**, 2866-2871.

Gagliardini, V., Fernandez, P.A., Lee, R.K.K., Drexler, H.C.A., Rotello, R.J., Fishman, M.C., and Yuan, J. (1994) Prevention of vertebrate neuronal death by the *CrmA* gene. *Science* **263**, 826-828.

Garcia-Martinez, V., Macias, D., Ganan, Y., Garcialobo, J.M., Francia, M.V., Fernandezteran, M.A., and Hurle, J.M. (1993) Internucleosomal DNA fragmentation and programmed cell death (apoptosis) in the interdigital tissue of the embryonic chick leg bud. *Journal Of Cell Science* **106**, 201-208.

Garkavtsev, I., Grigorian, I.A., Ossovskaya, V.S., Chernov, M.V., Chumakov, P.M., and Gudkov, A.V. (1998) The candidate tumour suppressor $p33^{ing1}$ cooperates with p53 in cell growth control. *Nature* **391**, 295-298.

Glucksmann, A. (1951) Cell deaths in normal vertebrate development. *Biological Review* **26**, 59-86.

Gluzman, Y. (1981) SV40-transformed simian cells support the replication of early SV40 mutants. *Cell* **23**, 175-182.

Golstein, P., Marguet, D., and Depraetere, V. (1995) Homology between reaper and the cell death domains of FAS and TNFR1. *Cell* **81**, 185-186.

Gottlieb, E., Haffner, R., Vonruden, T., Wagner, E.F., and Oren, M. (1994) Down-regulation of wild-type p53 activity interferes with apoptosis of IL-3-dependent hematopoietic cells following IL-3 withdrawal. *Embo Journal* **13**, 1368-1374.

Graeber, T.G., Peterson, J.F., Tsai, M., Monica, K., Fornace, A.J., and Giaccia, A.J. (1994) Hypoxia induces accumulation of p53 protein, but activation of a G_1 phase checkpoint by low oxygen conditions is independent of p53 status. *Molecular And Cellular Biology* **14**, 6264-6277.

Griffiths, S.D., Clarke, A.R., Healy, L.E., Ross, G., Ford, A.M., Hooper, M.L., Wyllie, A.H., and Greaves, M. (1997) Absence of p53 permits propagation of mutant cells following genotoxic damage. *Oncogene* **14**, 523-531.

Gu, Y., Turck, C.W., and Morgan, D.O. (1993) Inhibition of CDK2 activity *in vivo* by an associated 20K regulatory subunit. *Nature* **366**, 707-710.

Guillouf, C., Rosselli, F., Krishnaraju, K., Moustacchi, E., Hoffman, B., and Liebermann, D.A. (1995) p53 involvement in control of G_2 exit of the cell cycle - role in DNA damage-induced apoptosis. *Oncogene* **10**, 2263-2270.

Halazonetis, T.D., Davis, L.J., and Kandil, A.N. (1993) Wild-type p53 adopts a mutant-like conformation when bound to DNA. *Embo Journal* **12**, 1021-1028.

Hall, E.J. (1994) Radiobiology for the radiologist, 4th edition. J.B. Lippincott Company, Philadelphia, PA.

Han, Z.Y., Chatterjee, D., He, D.M., Early, J., Pantazis, P., Hendrickson, E.A., and Wyche, J.H. (1995) Evidence for a G_2 checkpoint in p53-independent apoptosis induction by X-ray irradiation. *Molecular Biology Of The Cell* **6**, 771

Harper, J.W., Adami, G.R., Wei, N., Keyomarsi, K., and Elledge, S.J. (1993) The p21 CDK-interacting protein CIP1 is a potent inhibitor of G_1 cyclin-dependent kinases. *Cell* **75**, 805-816.

Harper, J.W., Elledge, S.J., Keyomarsi, K., Dynlacht, B., Tsai, L.H., Zhang, P.M., Dobrowolski, S., Bai, C., Connell-Crowley, L., Swindell, E., Fox, M.P., and Wei, N. (1995) Inhibition of cyclin-dependent kinases by p21. *Molecular Biology Of The Cell* **6**, 387-400.

Harrington, E.A., Bennett, M.R., Fanidi, A., and Evan, G.I. (1994) c-MYC-induced apoptosis in fibroblasts is inhibited by specific cytokines. *Embo Journal* **13**, 3286-3295.

Harvey, M., Sands, A.T., Weiss, R.S., Hegi, M.E., Wiseman, R.W., Pantazis, P., Giovanella, B.C., Tainsky, M.A., Bradley, A., and Donehower, L.A. (1993) *In vitro* growth-characteristics of embryo fibroblasts isolated from *p53*-deficient mice. *Oncogene* **8**, 2457-2467.

Haupt, Y., Barak, Y., and Oren, M. (1996) Cell-type-specific inhibition of p53-mediated apoptosis by MDM2. *Embo Journal* **15**, 1596-1606.

Haupt, Y., Maya, R., Kazaz, A., and Oren, M. (1997) MDM2 promotes the rapid degradation of p53. *Nature* **387**, 296-299.

Heath, J.K., Smith, A.G., Hsu, L-W. and Rathjen, P.D. (1990) Growth and differentiation factors of pluripotential stem cells. *Journal of Cell Science Supplement* **13**, 75-85.

He, Z.G., Brinton, B.T., Greenblatt, J., Hassell, J.A., and Ingles, C.J. (1993) The transactivator protein VP16 and protein GAL4 bind replication factor A. *Cell* **73**, 1223-1232.

Held, K.D. (1997) Radiation-induced apoptosis and its relationship to loss of clonogenic survival. *Apoptosis* **2**, 265-282.

Hengartner, M.O., Ellis, R.E., and Horvitz, H.R. (1992) *Caenorhabditis elegans* gene *ced-9* protects cells from programmed cell-death. *Nature* **356**, 494-499.

Hermeking, H. and Eick, D. (1994) Mediation of c-MYC-induced apoptosis by p53. *Science* **265**, 2091-2093.

Hockenbery, D.M., Oltvai, Z.N., Yin, X.M., Milliman, C.L., and Korsmeyer, S.J. (1993) BCL-2 functions in an antioxidant pathway to prevent apoptosis. *Cell* **75**, 241-251.

Hofmann, K. and Bucher, P. (1997) The card domain: a new apoptotic signalling motif. *Trends In Biochemical Sciences* **22**, 155-156.

Hollstein, M., Rice, K., Greenblatt, M.S., Soussi, T., Fuchs, R., Sorlie, T., Hovig, E., Smithsorensen, B., Montesano, R., and Harris, C.C. (1994) Database of p53 gene somatic mutations in human tumors and cell-lines. *Nucleic Acids Research* **22**, 3551-3555.

Hooper, M.L. (1985) Mammalian cell genetics. John Wiley and Sons, Chichester.

Hooper, M., Hardy, K., Handyside, A., Hunter, S., and Monk, M. (1987) HPRT-deficient (Lesch-Nyhan) mouse embryos derived from germline colonization by cultured cells. *Nature* **326**, 292-295.

Hopcia, K.L., McCarey, Y.L., Sylvester, F.C., and Held, K.D. (1996) Radiation-induced apoptosis in HL-60 cells - oxygen effect, relationship between apoptosis and loss of clonogenicity, and dependence of time to apoptosis on radiation dose. *Radiation Research* **145**, 315-323.

Hopwood, D. and Levison, D.A. (1976) Atrophy and apoptosis in the cyclical human endometrium. *Journal of Pathology* **119**, 159-166.

Hsiang, Y.H., Hertzberg, R., Hecht, S., and Liu, L.F. (1985) Camptothecin induces protein-linked DNA breaks via mammalian DNA topoisomerase I. *Journal Of Biological Chemistry* **260**, 4873-4878.

Hugunin, M., Quintal, L.J., Mankovich, J.A., and Ghayur, T. (1996) Protease activity of *in vitro* transcribed and translated *Caenorhabditis elegans* cell death gene (*ced-3*) product. *Journal Of Biological Chemistry* **271**, 3517-3522.

Hunter, J.J. and Parslow, T.G. (1996) A peptide sequence from BAX that converts BCL-2 into an activator of apoptosis. *Journal Of Biological Chemistry* **271**, 8521-8524.

Hupp, T.R., Meek, D.W., Midgley, C.A., and Lane, D.P. (1992) Regulation of the specific DNA binding function of p53. *Cell* **71**, 875-886.

Hupp, T.R., Sparks, A., and Lane, D.P. (1995) Small peptides activate the latent sequence-specific DNA binding function of p53. *Cell* **83**, 237-245.

Irmler, M., Hofmann, K., Vaux, D., and Tschopp, J. (1997) Direct physical interaction between the *Caenorhabditis elegans* 'death proteins' CED-3 and CED-4. *Febs Letters* **406**, 189-190.

Ishizaki, K., Ejima, Y., Matsunaga, T., Hara, R., Sakamoto, A., Ikenaga, M., Ikawa, Y., and Aizawa, S. (1994) Increased UV-induced SCEs but normal repair of DNA damage in p53-deficient mouse cells. *International Journal Of Cancer* **58**, 254-257.

Jacks, T., Remington, L., Williams, B.O., Schmitt, E.M., Halachmi, S., Bronson, R.T., and Weinberg, R.A. (1994) Tumor spectrum analysis in p53 mutant mice. *Current Biology* **4**, 1-7.

Jackson, S.P. (1996) The recognition of DNA damage. *Current Opinion In Genetics & Development* **6**, 19-25.

Jacobson, M.D., Burne, J.F., King, M.P., Miyashita, T., Reed, J.C., and Raff, M.C. (1993) BCL-2 blocks apoptosis in cells lacking mitochondrial DNA. *Nature* **361**, 365-369.

Jacobson, M.D., Weil, M., and Raff, M.C. (1996) Role of CED-3/ICE-family proteases in staurosporine-induced programmed cell death. *Journal Of Cell Biology* **133**, 1041-1051.

James, C., Gschmeissner, S., Fraser, A., and Evan, G.I. (1997) CED-4 induces chromatin condensation in *Schizosaccharomyces pombe* and is inhibited by direct physical association with CED-9. *Current Biology* **7**, 246-252.

Jayaraman, L., Murthy, K.G.K., Zhu, C., Curran, T., Xanthoudakis, S., and Prives, C. (1997) Identification of redox/repair protein REF-1 as a potent activator of p53. *Genes & Development* **11**, 558-570.

Jayaraman, L. and Prives, C. (1995) Activation of p53 sequence-specific DNA binding by short single strands of DNA requires the p53 C-terminus. *Cell* **81**, 1021-1029.

Johnson, P., Chung, S., and Benchimol, S. (1993) Growth suppression of Friend virus-transformed erythroleukemia cells by p53 protein is accompanied by hemoglobin production and is sensitive to erythropoietin. *Molecular And Cellular Biology* **13**, 1456-1463.

Jost, C.A., Marin, M.C., and Kaelin, W.G. (1997) p73 is a human p53-related protein that can induce apoptosis. *Nature* **389**, 191-194.

Kaghad, M., Bonnet, H., Yang, A., Creancier, L., Biscan, J.C., Valent, A., Minty, A., Chalon, P., Lelias, J.M., Dumont, X., Ferrara, P., McKeon, F., and Caput, D. (1997) Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers. *Cell* **90**, 809-819.

Kamada, S., Shimono, A., Shinto, Y., Tsujimura, T., Takahashi, T., Noda, T., Kitamura, Y., Kondoh, H., and Tsujimoto, Y. (1995) BCL-2 deficiency in mice leads to pleiotropic abnormalities -accelerated lymphoid cell death in thymus and spleen, polycystic kidney, hair hypopigmentation, and distorted small intestine. *Cancer Research* **55**, 354-359.

Kastan, M.B., Onyekwere, O., Sidransky, D., Vogelstein, B., and Craig, R.W. (1991) Participation of p53 protein in the cellular response to DNA damage. *Cancer Research* **51**, 6304-6311.

Kazantsev, A. and Sancar, A. (1995) Does the p53 up-regulated GADD45 protein have a role in excision repair? *Science* **270**, 1003-1004.

Kern, S.E., Kinzler, K.W., Bruskin, A., Jarosz, D., Friedman, P., Prives, C., and Vogelstein, B. (1991) Identification of p53 as a sequence-specific DNA binding protein. *Science* **252**, 1708-1711.

Kerr, J.F.R. (1971) Shrinkage necrosis: a distinct mode of cellular death. *Journal of Pathology* **105**, 13-20.

Kerr, J.F.R, Searle, J., Harmon, B.V. and Bishop, C.J. (1987) Apoptosis. *In: Perspectives on Mammalian Cell Death. Potten, C.S. (ed.)* Oxford University Press. 93.

Kerr, J.F.R., Wyllie, A.H. and Currie, A.R. (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *British Journal of Cancer* **26**, 239-257.

Kluck, R.M., BossyWetzel, E., Green, D.R., and Newmeyer, D.D. (1997) The release of cytochrome c from mitochondria: a primary site for BCL2 regulation of apoptosis. *Science* **275**, 1132-1136.

Knudson, C.M., Tung, K.S.K., Tourtellotte, W.G., Brown, G.A.J., and Korsmeyer, S.J. (1995) *Bax*-deficient mice with lymphoid hyperplasia and male germ cell death. *Science* **270**, 96-99.

Ko, L.J. and Prives, C. (1996) p53 - puzzle and paradigm. *Genes & Development* **10**, 1054-1072.

Kolodner, R. (1996) Biochemistry and genetics of eukaryotic mismatch repair. *Genes & Development* **10**, 1433-1442.

Krajewski, S., Tanaka, S., Takayama, S., Schibler, M.J., Fenton, W., and Reed, J.C. (1993) Investigation of the subcellular distribution of the BCL2 oncoprotein - residence in the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membranes. *Cancer Research* **53**, 4701-4714.

Kroemer, G., Petit, P., Zamzami, N., Vayssiere, J.L., and Mignotte, B. (1995) The biochemistry of programmed cell death. *Faseb Journal* **9**, 1277-1287.

Kroemer, G. (1997) The proto-oncogene *Bcl2* and its role in regulating apoptosis. *Nature Medicine* **3**, 614-620.

Kroemer, G., Zamzami, N., and Susin, S.A. (1997) Mitochondrial control of apoptosis. *Immunology Today* **18**, 44-51.

Kubbutat, M.H.G., Jones, S.N., and Vousden, K.H. (1997) Regulation of p53 stability by MDM2. *Nature* **387**, 299-303.

Kuerbitz, S.J., Plunkett, B.S., Walsh, W.V., and Kastan, M.B. (1992) Wild-type p53 is a cell cycle checkpoint determinant following irradiation. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **89**, 7491-7495.

Kuida, K., Lippke, J.A., Ku, G., Harding, M.W., Livingston, D.J., Su, M.S.S., and Flavell, R.A. (1995) Altered cytokine export and apoptosis in mice deficient in interleukin-1-beta converting enzyme. *Science* **267**, 2000-2003.

Kuida, K., Zheng, T.S., Na, S.Q., Kuan, C.Y., Yang, D., Karasuyama, H., Rakic, P., and Flavell, R.A. (1996) Decreased apoptosis in the brain and premature lethality in CPP32- deficient mice. *Nature* **384**, 368-372.

Kuleszmartin, M.F., Lisafeld, B., Huang, H., Kisiel, N.D., and Lee, L. (1994) Endogenous p53 protein generated from wild-type alternatively spliced p53 RNA in mouse epidermal cells. *Molecular And Cellular Biology* **14**, 1698-1708.

Lane, D.P. (1992) Cancer - p53, guardian of the genome. *Nature* **358**, 15-16.

Langley, R.E., Palayoor, S.T., Coleman, C.N., and Bump, E.A. (1994) Radiation induced apoptosis in F9 teratocarcinoma cells. *International Journal Of Radiation Biology* **65**, 605-610.

Lazebnik, Y.A., Cole, S., Cooke, C.A., Nelson, W.G., and Earnshaw, W.C. (1993) Nuclear events of apoptosis *in vitro* in cell-free mitotic extracts - a model system for analysis of the active phase of apoptosis. *Journal Of Cell Biology* **123**, 7-22.

Lazebnik, Y.A., Kaufmann, S.H., Desnoyers, S., Poirier, G.G., and Earnshaw, W.C. (1994) Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature* **371**, 346-347.

Lee, S., Elenbaas, B., Levine, A., and Griffith, J. (1995) p53 and its 14 kDa C-terminal domain recognize primary DNA damage in the form of insertion deletion mismatches. *Cell* **81**, 1013-1020.

Leveillard, T., Andera, L., Bissonnette, N., Schaeffer, L., Bracco, L., Egly, J.M., and Wasylyk, B. (1996) Functional interactions between p53 and the TFIIH complex are affected by tumor-associated mutations. *Embo Journal* **15**, 1615-1624.

Li, G., Mitchell, D.L., Ho, V.C., Reed, J.C., and Tron, V.A. (1996) Decreased DNA repair but normal apoptosis in ultraviolet irradiated skin of *p53* transgenic mice. *American Journal Of Pathology* **148**, 1113-1123.

Li, R., Waga, S., Hannon, G.J., Beach, D., and Stillman, B. (1994) Differential effects by the p21 CDK inhibitor on PCNA-dependent DNA replication and repair. *Nature* **371**, 534-537.

Li, R. and Botchan, M.R. (1993) The acidic transcriptional activation domains of VP16 and p53 bind the cellular replication protein-A and stimulate *in vitro* BPV-1 DNA replication. *Cell* **73**, 1207-1221.

Lin, J.Y., Chen, J.D., Elenbaas, B., and Levine, A.J. (1994) Several hydrophobic amino acids in the p53 amino terminal domain are required for transcriptional activation, binding to MDM-2 and the adenovirus-5 E1B 55-kD protein. *Genes & Development* **8**, 1235-1246.

Ling, C.C., Guo, M., Chen, C.H., and Deloherey, T. (1995) Radiation induced apoptosis - effects of cell age and dose fractionation. *Cancer Research* **55**, 5207-5212.

Linke, S.P., Clarkin, K.C., and Wahl, G.M. (1997) p53 mediates permanent arrest over multiple cell cycles in response to gamma irradiation. *Cancer Research* **57**, 1171-1179.

Lippke, J.A., Gordon, L.K., Brash, D.E., and Haseltine, W.A. (1981) Distribution of UV light-induced damage in a defined sequence of human DNA - detection of alkaline-sensitive lesions at pyrimidine nucleoside cytidine sequences. *Proceedings Of The National Academy Of Sciences Of The United States Of America-Biological Sciences* **78**, 3388-3392.

Liu, X., Miller, C.W., Koeffler, P.H., and Berk, A.J. (1993) The p53 activation domain binds the TATA box binding polypeptide in holo-TFIID, and a neighboring p53 domain inhibits transcription. *Molecular And Cellular Biology* **13**, 3291-3300.

Liu, X.S., Kim, C.N., Yang, J., Jemmerson, R., and Wang, X.D. (1996) Induction of apoptotic program in cell-free extracts -requirement for dATP and cytochrome c. *Cell* **86**, 147-157.

Livingstone, L.R., White, A., Sprouse, J., Livanos, E., Jacks, T., and Tisty, T.D. (1992) Altered cell cycle arrest and gene amplification potential accompany loss of wild-type p53. *Cell* **70**, 923-935.

Long, B.H., Musial, S.T., and Brattain, M.G. (1984) Comparison of cytotoxicity and DNA breakage activity of congeners of podophyllotoxin including VP16-213 and VM26 - a quantitative structure-activity relationship. *Biochemistry* **23**, 1183-1188.

Longthorne, V.L. and Williams, G.T. (1997) Caspase activity is required for commitment to FAS-mediated apoptosis. *Embo Journal* **16**, 3805-3812.

Lowe, S.W., Schmitt, E.M., Smith, S.W., Osborne, B.A., and Jacks, T. (1993) p53 is required for radiation-induced apoptosis in mouse thymocytes. *Nature* **362**, 847-849.

Lu, H. and Levine, A.J. (1995) Human TAF(II)31 protein is a transcriptional coactivator of the p53 protein. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **92**, 5154-5158.

Lu, X. and Lane, D.P. (1993) Differential induction of transcriptionally active p53 following UV or ionizing-radiation - defects in chromosome instability syndromes. *Cell* **75**, 765-778.

Lutzker, S.G. and Levine, A.J. (1996) A functionally inactive p53 protein in teratocarcinoma cells is activated by either DNA damage or cellular differentiation. *Nature Medicine* **2**, 804-810.

Lyons, S.K. and Clarke, A.R. (1997) Apoptosis and carcinogenesis. *British Medical Bulletin* **53** (No.3), 554-569.

MacCallum, D.H., Hupp, T.R., Midgley, C.A., Stuart, D., Campbell, S.J., Harper, A., Walsh, F.S., Wright, E.G., Balmain, A., Lane, D.P. and Hall, P.A. (1996) The p53 response to ionising radiation in adult and developing murine tissues. *Oncogene* **13**, 2575-2587.

Mack, D.H., Vartikar, J., Pipas, J.M., and Laimins, L.A. (1993) Specific repression of TATA-mediated but not initiator-mediated transcription by wild-type p53. *Nature* **363**, 281-283.

Magin, T.M., McWhir, J., and Melton, D.W. (1992) A new mouse embryonic stem cell line with good germ line contribution and gene targeting frequency. *Nucleic Acids Research* **20**, 3795

Maheswaran, S., Englert, C., Bennett, P., Heinrich, G., and Haber, D.A. (1995) The *WT1* gene-product stabilizes p53 and inhibits p53-mediated apoptosis. *Genes & Development* **9**, 2143-2156.

Maltzman, W. and Czyzyk, L. (1984) UV irradiation stimulates levels of p53 cellular tumor-antigen in nontransformed mouse cells. *Molecular And Cellular Biology* **4**, 1689-1694.

Mayr, G.A., Reed, M., Wang, P., Wang, Y., Schwedes, J.F., and Tegtmeyer, P. (1995) Serine phosphorylation in the NH2 terminus of p53 facilitates transactivation. *Cancer Research* **55**, 2410-2417.

McBurney, M.W. (1976) Clonal lines of teratocarcinoma cells *in vitro*: Differentiation and cytogenetic characteristics. *Journal of Cell Physiology* **89**, 441-456.

McBurney, M.W. and Rogers, B.J. (1982) Isolation of male embryonal carcinoma cell lines and their chromosome replication patterns. *Developmental Biology* **89**, 503-508.

McCormick, F., Clark, R., Harlow, E., and Tjian, R. (1981) SV40 T antigen binds specifically to a cellular 53k protein *in vitro*. *Nature* **292**, 63-65.

Meek, D.W. (1994) Posttranslational modification of p53. *Seminars In Cancer Biology* **5**, 203-210.

Meling, G.I., Lothe, R.A., Borresen, A.L., Graue, C., Hauge, S., Clausen, O.P.F., and Rognum, T.O. (1993) The TP53 tumor suppressor gene in colorectal carcinomas .2. relation to DNA ploidy pattern and clinicopathological variables. *British Journal Of Cancer* **67**, 93-98.

Merritt, A.J., Potten, C.S., Kemp, C.J., Hickman, J.A., Balmain, A., Lane, D.P., and Hall, P.A. (1994) The role of p53 in spontaneous and radiation induced apoptosis in the gastrointestinal tract of normal and p53-deficient mice. *Cancer Research* **54**, 614-617.

Midgley, C.A., Owens, B., Briscoe, C.V., Thomas, D.B., Lane, D.P., and Hall, P.A. (1995) Coupling between gamma irradiation, p53 induction and the apoptotic response depends upon cell type *in vivo*. *Journal Of Cell Science* **108**, 1843-1848.

Miller, J.F.A.P. and Heath, W.R. (1993) Self-ignorance in the peripheral T-cell pool. *Immunological Reviews* **133**, 131-150.

Milligan, C.E. and Schwartz, L.M. (1997) Programmed cell death during animal development. *British Medical Bulletin* **52** (No.3), 570-590.

Miura, M. and Yuan, J.Y. (1996) Mechanisms of programmed cell death in *Caenorhabditis elegans* and vertebrates. *Current Topics In Developmental Biology* **32**, 139-174.

Miyashita, T. and Reed, J.C. (1995) Tumor suppressor p53 is a direct transcriptional activator of the human *BAX* gene. *Cell* **80**, 293-299.

Momand, J., Zambetti, G.P., Olson, D.C., George, D., and Levine, A.J. (1992) The MDM2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. *Cell* **69**, 1237-1245.

Monaghan, P., Robertson, D., Amos, T.A.S., Dyer, M.J.S., Mason, D.Y., and Greaves, M.F. (1992) Ultrastructural localization of BCL2 protein. *Journal Of Histochemistry & Cytochemistry* **40**, 1819-1825.

Morgenbesser, S.D., Williams, B.O., Jacks, T., and Depinho, R.A. (1994) p53-dependent apoptosis produced by *Rb*-deficiency in the developing mouse lens. *Nature* **371**, 72-74.

Mori, C., Nakamura, N., Okamoto, Y., Osawa, M., and Shiota, K. (1994) Cytochemical identification of programmed cell death in the fusing fetal mouse palate by specific labeling of DNA fragmentation. *Anatomy And Embryology* **190**, 21-28.

Mortensen, R.M., Conner, D.A., Chao, S., Geisterferlowrance, A.A.T., and Seidman, J.G. (1992) Production of homozygous mutant ES cells with a single targeting construct. *Molecular And Cellular Biology* **12**, 2391-2395.

Mosner, J., Mummenbrauer, T., Bauer, C., Sczakiel, G., Grosse, F., and Deppert, W. (1995) Negative feedback regulation of wild-type p53 biosynthesis. *Embo Journal* **14**, 4442-4449.

Motoyama, N., Wang, F.P., Roth, K.A., Sawa, H., Nakayama, K., Negishi, I., Senju, S., Zhang, Q., Fujii, S., and Loh, D.Y. (1995) Massive cell death of immature hematopoietic cells and neurons in BCLX deficient mice. *Science* **267**, 1506-1510.

Mountford, P., Zevnik, B., Duwel, A., Nichols, J., Li, M., Dani, C., Robertson, M., Chambers, I., and Smith, A. (1994) Dicistronic targeting constructs - reporters and modifiers of mammalian gene expression. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **91**, 4303-4307.

Muchmore, S.W., Sattler, M., Liang, H., Meadows, R.P., Harlan, J.E., Yoon, H.S., Nettesheim, D., Chang, B.S., Thompson, C.B., Wong, S.L., Ng, S.C., and Fesik, S.W. (1996) X-ray and NMR structure of human BCLX(L), an inhibitor of programmed cell death. *Nature* **381**, 335-341.

Muzio, M., Chinnaiyan, A.M., Kischkel, F.C., Orourke, K., Shevchenko, A., Ni, J., Scaffidi, C., Bretz, J.D., Zhang, M., Gentz, R., Mann, M., Krammer, P.H., Peter, M.E., and Dixit, V.M. (1996) FLICE, a novel FADD homologous ICE/CED-3-like protease, is recruited to the CD95 (FAS/APO-1) death-inducing signaling complex. *Cell* **85**, 817-827.

Nakayama, K., Negishi, I., Kuida, K., Sawa, H., and Loh, D.Y. (1994) Targeted disruption of *Bcl2*-alpha-beta in mice - occurrence of gray hair, polycystic kidney disease, and lymphocytopenia. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **91**, 3700-3704.

Naumovski, L. and Cleary, M.L. (1996) The p53-binding protein 53BP2 also interacts with BCL2 and impedes cell cycle progression at G_2/M. *Molecular And Cellular Biology* **16**, 3884-3892.

Nelson, E.M., Tewey, K.M., and Liu, L.F. (1984) Mechanism of antitumor drug action - poisoning of mammalian DNA topoisomerase II on DNA by 4'-(9-acridinylamino)-methanesulfon-meta- anisidide. *Proceedings Of The National Academy Of Sciences Of The United States Of America-Biological Sciences* **81**, 1361-1365.

Nelson, W.G. and Kastan, M.B. (1994) DNA strand breaks - the DNA template alterations that trigger p53-dependent DNA damage response pathways. *Molecular And Cellular Biology* **14**, 1815-1823.

Nemazee, D.A. and Burki, K. (1989) Clonal deletion of lymphocyte-B in a transgenic mouse bearing anti- MHC class-I antibody genes. *Nature* **337**, 562-566.

Nicholson, D.W., Ali, A., Thornberry, N.A., Vaillancourt, J.P., Ding, C.K., Gallant, M., Gareau, Y., Griffin, P.R., Labelle, M., Lazebnik, Y.A., Munday, N.A., Raju, S.M., Smulson, M.E., Yamin, T.T., Yu, V.L., and Miller, D.K. (1995) Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature* **376**, 37-43.

Nishino, H., Knoll, A., Buettner, V.L., Frisk, C.S., Maruta, Y., Haavik, J., and Sommer, S.S. (1995) *p53* wild-type and *p53* nullizygous big blue transgenic mice have similar frequencies and patterns of observed mutation in liver, spleen and brain. *Oncogene* **11**, 263-270.

Oberosler, P., Hloch, P., Ramsperger, U., and Stahl, H. (1993) p53-catalyzed annealing of complementary single stranded nucleic acids. *Embo Journal* **12**, 2389-2396.

Okamoto, K. and Beach, D. (1994) Cyclin G is a transcriptional target of the p53 tumor suppressor protein. *Embo Journal* **13**, 4816-4822.

Oliner, J.D., Pietenpol, J.A., Thiagalingam, S., Gvuris, J., Kinzler, K.W., and Vogelstein, B. (1993) Oncoprotein MDM2 conceals the activation domain of tumor suppressor p53. *Nature* **362**, 857-860.

Olive, P.L., Banath, J.P., and Durand, R.E. (1996) Development of apoptosis and polyploidy in human lymphoblast cells as a function of position in the cell cycle at the time of irradiation. *Radiation Research* **146**, 595-602.

Oltvai, Z.N., Milliman, C.L., and Korsmeyer, S.J. (1993) BCL2 heterodimerizes *in vivo* with a conserved homolog, BAX, that accelerates programmed cell death. *Cell* **74**, 609-619.

O'Reilly, J.P. and Mothersill, C. (1997) Comparative effects of UV A and UV B on clonogenic survival and delayed cell death in skin cell lines from humans and fish. *International Journal Of Radiation Biology* **72**, 111-119.

Oren, M. (1994) Relationship of p53 to the control of apoptotic cell death. *Seminars In Cancer Biology* **5**, 221-227.

Orth, K., Chinnaiyan, A.M., Garg, M., Froelich, C.J., and Dixit, V.M. (1996) The CED-3/ICE-like protease MCH2 is activated during apoptosis and cleaves the death substrate lamin A. *Journal Of Biological Chemistry* **271**, 16443-16446.

Perry, M.E., Piette, J., Zawadzki, J.A., Harvey, D., and Levine, A.J. (1993) The *Mdm2* gene is induced in response to UV light in a p53-dependent manner. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **90**, 11623-11627.

Powell, S. and McMillan, T.J. (1990) DNA damage and repair following treatment with ionizing radiation. *Radiotherapy And Oncology* **19**, 95-108.

Powell, S.N., DeFrank, J.S., Connell, P., Eogan, M., Preffer, F., Dombkowski, D., Tang, W., and Friend, S. (1995) Differential sensitivity of $p53^-$ and $p53^+$ cells to caffeine-induced radiosensitization and override of G_2 delay. *Cancer Research* **55**, 1643-1648.

Pratt, R.M. and Greene, R.M. (1976) Inhibition of palatal epithelial cell death by altered protein synthesis. *Developmental Biology* **54**, 135-145.

Prelich, G., Kostura, M., Marshak, D.R., Mathews, M.B., and Stillman, B. (1987) The cell cycle regulated proliferating cell nuclear antigen is required for SV40 DNA replication *in vitro*. *Nature* **326**, 471-475.

Prost, S., Bellamy, C.O.C., Clarke, A.R., Wyllie, A.H. and Harrison, D.J. (1998) p53-independent DNA repair and cell cycle arrest in embryonic stem cells. *FEBS letters* **425**, 499-504.

Purdie, C.A. (1993) The role of p53 in colorectal carcinogenesis. *PhD thesis, University of Edinburgh.*

Purdie, C.A., Harrison, D.J., Peter, A., Dobbie, L., White, S., Howie, S.E.M., Salter, D.M., Bird, C.C., Wyllie, A.H., Hooper, M.L., and Clarke, A.R. (1994) Tumor incidence, spectrum and ploidy in mice with a large deletion in the *p53* gene. *Oncogene* **9**, 603-609.

Qin, X.Q., Livingston, D.M., Kaelin, W.G., and Adams, P.D. (1994) Deregulated transcription factor E2F-1 expression leads to S-phase entry and p53-mediated apoptosis. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **91**, 10918-10922.

Radford, I.R. (1985) The level of induced DNA double strand breakage correlates with cell killing after X-irradiation. *International Journal Of Radiation Biology* **48**, 45-54.

Radford, I.R. and Murphy, T.K. (1994) Radiation response of mouse lymphoid and myeloid cell lines .3. different signals can lead to apoptosis and may influence sensitivity to killing by DNA double strand breakage. *International Journal Of Radiation Biology* **65**, 229-239.

Rasko, I., Georgieva, M., Farkas, G., Santha, M., Coates, J., Burg, K., Mitchell, D.L., and Johnson, R.T. (1993) New patterns of bulk DNA repair in ultraviolet irradiated mouse embryo carcinoma cells following differentiation. *Somatic Cell And Molecular Genetics* **19**, 245-255.

Raulet, D.H., Garman, R.D., Saito, H., and Tonegawa, S. (1985) Developmental regulation of T-cell receptor gene expression. *Nature* **314**, 103-107.

Reed, M., Woelker, B., Wang, P., Wang, Y., Anderson, M.E., and Tegtmeyer, P. (1995) The C-terminal domain of p53 recognizes DNA damaged by ionizing radiation. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **92**, 9455-9459.

Renzing, J. and Lane, D.P. (1995) p53-dependent growth arrest following calcium phosphate-mediated transfection of murine fibroblasts. *Oncogene* **10**, 1865-1868.

Robertson, E.J. and Bradley, A. (1986) Chapter 15: Production of permanent cell lines from early embryos and their use in studying developmental problems. *In: Experimental approaches to mammalian embryonic development. Rossant, J. and Dederson, R.A. (eds.).* Cambridge University Press, Cambridge, 475-508.

Robins, P., Jones, C.J., Biggerstaff, M., Lindahl, T., and Wood, R.D. (1991) Complementation of DNA repair in xeroderma pigmentosum group A cell extracts by a protein with affinity for damaged DNA. *Embo Journal* **10**, 3913-3921.

Rogel, A., Popliker, M., Webb, C.G., and Oren, M. (1985) p53 cellular tumor-antigen - analysis of messenger RNA levels in normal adult tissues, embryos, and tumors. *Molecular And Cellular Biology* **5**, 2851-2855.

Rouvier, E., Luciani, M.F., and Golstein, P. (1993) FAS involvement in Ca^{2+}-independent T cell mediated cytotoxicity. *Journal Of Experimental Medicine* **177**, 195-200.

Russell, J., Wheldon, T.E., and Stanton, P. (1995) Radioresistant variant derived from a human neuroblastoma cell line is less prone to radiation induced apoptosis. *Cancer Research* **55**, 4915-4921.

Ryan, J.J., Danish, R., Gottlieb, C.A., and Clarke, M.F. (1993) Cell cycle analysis of p53-induced cell death in murine erythroleukemia cells. *Molecular And Cellular Biology* **13**, 711-719.

Sabapathy, K., Klemm, M., Jaenisch, R., and Wagner, E.F. (1997) Regulation of ES cell differentiation by functional and conformational modulation of p53. *Embo Journal* **16**, 6217-6229.

Sabbatini, P., Chiou, S.K., Rao, L., and White, E. (1995) Modulation of p53-mediated transcriptional repression and apoptosis by the adenovirus E1B 19k protein. *Molecular And Cellular Biology* **15**, 1060-1070.

Sah, V.P., Attardi, L.D., Mulligan, G.J., Williams, B.O., Bronson, R.T., and Jacks, T. (1995) A subset of p53-deficient embryos exhibit exencephaly. *Nature Genetics* **10**, 175-180.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular cloning: A laboratory manual. Cold Spring Harbour Press Laboratory Press, New York.

Sands, A.T., Suraokar, M.B., Sanchez, A., Marth, J.E., Donehower, L.A., and Bradley, A. (1995) p53 deficiency does not affect the accumulation of point mutations in a transgene target. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **92**, 8517-8521.

Sang, B.C., Chen, J.Y., Minna, J., and Barbosa, M.S. (1994) Distinct regions of p53 have a differential role in transcriptional activation and repression functions. *Oncogene* **9**, 853-859.

Sankaranarayanan, K. (1991) Ionizing radiation and genetic risks .3. nature of spontaneous and radiation induced mutations in mammalian *in vitro* systems and mechanisms of induction of mutations by radiation. *Mutation Research* **258**, 75-97.

Saunders, J.W. and Fallon J.S. (1966) Cell death in morphogenesis. *In: Lock, M. (ed.) Major problems in developmental biology.* 25th Symposium of the Society for Developmental Biology, New York Academic Press, 289-314.

Schlegel, J., Peters, I., Orrenius, S., Miller, D.K., Thornberry, N.A., Yamin, T.T., and Nicholson, D.W. (1996) CPP32 apopain is a key interleukin-1-beta converting enzyme-like protease involved in FAS-mediated apoptosis. *Journal Of Biological Chemistry* **271**, 1841-1844.

Setlow, R.B. and Carrier, W.L. (1966) Pyrimidine dimers in ultraviolet irradiated DNAs. *Journal of Molecular Biology* **17**, 237-254.

Seto, E., Usheva, A., Zambetti, G.P., Momand, J., Horikoshi, N., Weinmann, R., Levine, A.J., and Shenk, T. (1992) Wild-type p53 binds to the TATA-binding protein and represses transcription. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **89**, 12028-12032.

Seymour, C. and Mothersill, C. (1992) All colonies of CHO-K1 cells surviving gamma irradiation contain nonviable cells. *Mutation Research* **267**, 19-30.

Seymour, C.B., Mothersill, C. and Alper, T. (1986) High yields of lethal mutations in somatic mammalian cells that survive ionising radiation. *Journal of Radiation Biology* **53**, 319-330.

Shan, B. and Lee, W.H. (1994) Deregulated expression of E2F-1 induces S-phase entry and leads to apoptosis. *Molecular And Cellular Biology* **14**, 8166-8173.

Shaulian, E., Haviv, I., Shaul, Y., and Oren, M. (1995) Transcriptional repression by the C-terminal domain of p53. *Oncogene* **10**, 671-680.

Shaulsky, G., Goldfinger, N., Peled, A., and Rotter, V. (1991) Involvement of wild-type p53 in pre-B-cell differentiation *in vitro*. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **88**, 8982-8986.

Shibasaki, F., Kondo, E., Akagi, T., and McKeon, F. (1997) Suppression of signalling through transcription factor NF-AT by interactions between calcineurin and BCL2. *Nature* **386**, 728-731.

Shiio, Y., Yamamoto, T., and Yamaguchi, N. (1992) Negative regulation of RB expression by the *p53* gene product. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **89**, 5206-5210.

Slebos, R.J.C., Lee, M.H., Plunkett, B.S., Kessis, T.D., Williams, B.O., Jacks, T., Hedrick, L., Kastan, M.B., and Cho, K.R. (1994) p53-dependent G_1 arrest involves pRB-related proteins and is disrupted by the human papillomavirus-16-E7 oncoprotein. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **91**, 5320-5324.

Slee, E.A., Zhu, H.J., Chow, S.C., MacFarlane, M., Nicholson, D.W., and Cohen, G.M. (1996) Benzyloxycarbonyl-val-ala-asp (OME) fluoromethylketone (Z-VAD.fmk) inhibits apoptosis by blocking the processing of CPP32. *Biochemical Journal* **315**, 21-24.

Smith, A.G., Heath, J.K., Donaldson, D.D., Wong, G.G., Moreau, J., Stahl, M., and Rogers, D. (1988) Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. *Nature* **336**, 688-690.

Smith, M.L., Chen, I.T., Zhan, Q.M., Bae, I.S., Chen, C.Y., Gilmer, T.M., Kastan, M.B., O'Connor, P.M., and Fornace, A.J. (1994) Interaction of the p53 regulated protein GADD45 with proliferating cell nuclear antigen. *Science* **266**, 1376-1380.

Smith, M.L., Chen, I.T., Zhan, Q.M., O'Connor, P.M., and Fornace, A.J. (1995) Involvement of the p53 tumor suppressor in repair of UV-type DNA damage. *Oncogene* **10**, 1053-1059.

Soddu, S., Blandino, G., Citro, G., Scardigli, R., Piaggio, G., Ferber, A., Calabretta, B., and Sacchi, A. (1994) Wild-type p53 gene expression induces granulocytic differentiation of HL-60 cells. *Blood* **83**, 2230-2237.

Soddu, S., Blandino, G., Scardigli, R., Coen, S., Marchetti, A., Rizzo, M.G., Bossi, G., Cimino, L., Crescenzi, M., and Sacchi, A. (1996) Interference with p53 protein inhibits hematopoietic and muscle differentiation. *Journal Of Cell Biology* **134**, 193-204.

Soussi, T., Defromentel, C.C., and May, P. (1990) Structural aspects of the p53 protein in relation to gene evolution. *Oncogene* **5**, 945-952.

Spector, M.S., Desnoyers, S., Hoeppner, D.J., and Hengartner, M.O. (1997) Interaction between the *C elegans* cell death regulators CED-9 and CED-4. *Nature* **385**, 653-656.

Stewart, N., Hicks, G.G., Paraskevas, F., and Mowat, M. (1995) Evidence for a 2nd cell cycle block at G_2/M by p53. *Oncogene* **10**, 109-115.

Story, M.D., Voehringer, D.W., Malone, C.G., Hobbs, M.L and Meyn, R.E. (1994) Radiation induced apoptosis in sensitive and resistant cells isolated from a mouse lymphoma. *International Journal of Radiation Biology* **66**, 659-668.

Strange, R., Li, F., Saurer, S., Burkhardt, A., and Friis, R.R. (1992) Apoptotic cell death and tissue remodeling during mouse mammary gland involution. *Development* **115**, 49-58.

Subler, M.A., Martin, D.W., and Deb, S. (1994) Overlapping domains on the p53 protein regulate its transcriptional activation and repression functions. *Oncogene* **9**, 1351-1359.

Sugano, T., Nitta, M., Ohmori, H., and Yamaizumi, M. (1995) Nuclear accumulation of p53 in normal human fibroblasts is induced by various cellular stresses which evoke the heart shock response, independently of the cell cycle. *Japanese Journal Of Cancer Research* **86**, 415-418.

Susin, S.A., Zamzami, N., Castedo, M., Hirsch, T., Marchetti, P., Macho, A., Daugas, E., Geuskens, M., and Kroemer, G. (1996) BCL2 inhibits the mitochondrial release of an apoptogenic protease. *Journal Of Experimental Medicine* **184**, 1331-1341.

Takenaka, I., Morin, F., Seizinger, B.R., and Kley, N. (1995) Regulation of the sequence-specific DNA binding function of p53 by protein kinase-C and protein phosphatases. *Journal Of Biological Chemistry* **270**, 5405-5411.

Tamura, T., Ishihara, M., Lamphier, M.S., Tanaka, N., Oishi, I., Aizawa, S., Matsuyama, T., Mak, T.W., Taki, S., and Taniguchi, T. (1995) An IRF-1-dependent pathway of DNA damage-induced apoptosis in mitogen-activated T lymphocytes. *Nature* **376**, 596-599.

Tanaka, N., Ishihara, M., Lamphier, M.S., Nozawa, H., Matsuyama, T., Mak, T.W., Aizawa, S., Tokino, T., Oren, M., and Taniguchi, T. (1996) Cooperation of the tumor suppressors IRF-1 and p53 in response to DNA damage. *Nature* **382**, 816-818.

Tanaka, S., Saito, K., and Reed, J.C. (1993) Structure-function analysis of the *BCL2* oncoprotein - addition of a heterologous transmembrane domain to portions of the BL2-beta protein restores function as a regulator of cell survival. *Journal Of Biological Chemistry* **268**, 10920-10926.

Tauchi, H. and Sawada, S. (1994) Analysis of mitotic cell death caused by radiation in mouse leukemia l5178Y cells - apoptosis is the ultimate form of cell death following mitotic failure. *International Journal Of Radiation Biology* **65**, 449-455.

Thomas, K.R. and Capecchi, M.R. (1987) Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. *Cell* **51**, 503-512.

Thompson, L.H. and Suit, H.D. (1969) Proliferation kinetics of mouse L cells with time-lapse photography II. *International Journal of Radiation Biology* **15**, 347-362.

Thompson, S., Clarke, A.R., Pow, A.M., Hooper, M.L., and Melton, D.W. (1989) Germ line transmission and expression of a corrected *Hprt* gene produced by gene targeting in embryonic stem cells. *Cell* **56**, 313-321.

Thornberry, N.A. (1996) The caspase family of cysteine proteases. *British Medical Bulletin* **53** (No.3) 478-490.

Thornberry, N.A., Bull, H.G., Calaycay, J.R., Chapman, K.T., Howard, A.D., Kostura, M.J., Miller, D.K., Molineaux, S.M., Weidner, J.R., Aunins, J., Elliston, K.O., Ayala, J.M., Casano, F.J., Chin, J., Ding, G.J.F., Egger, L.A., Gaffney, E.P., Limjuco, G., Palyha, O.C., Raju, S.M., Rolando, A.M., Salley, J.P., Yamin, T.T., Lee, T.D., Shively, J.E., MacCross, M., Mumford, R.A., Schmidt, J.A., and Tocci, M.J. (1992) A novel heterodimeric cysteine protease is required for interleukin- 1-beta processing in monocytes. *Nature* **356**, 768-774.

Thornberry, N.A., Rosen, A. and Nicholson, D.W. (1997) Control of apoptosis by proteases. *Advances in Pharmacology* **41**.

Thut, C.J., Chen, J.L., Klemm, R., and Tjian, R. (1995) p53 transcriptional activation mediated by coactivators TAF(II)40 and TAF(II)60. *Science* **267**, 100-104.

Tonegawa, S. (1983) Somatic generation of antibody diversity. *Nature* **302**, 575-581.

Tong, X., Drapkin, R., Reinberg, D., and Kieff, E. (1995) The 62-kDa and 80-kDa subunits of transcription factor IIH mediate the interaction with Epstein Barr virus nuclear protein-2. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **92**, 3259-3263.

Tsujimoto, Y., Cossman, J., Jaffe, E., and Croce, C.M. (1985) Involvement of the *BCL2* gene in human follicular lymphoma. *Science* **228**, 1440-1443.

Tsukada, T., Tomooka, Y., Takai, S., Ueda, Y., Nishikawa, S., Yagi, T., Tokunaga, T., Takeda, N., Suda, Y., Abe, S., Matsuo, I., Ikawa, Y., and Aizawa, S. (1993) Enhanced proliferative potential in culture of cells from p53-deficient mice. *Oncogene* **8**, 3313-3322.

Uhlmann, E.J., Dsaeipper, C., Subramanian, T., Wagner, A.J., Hay, N., and Chinnadurai, G. (1996) Deletion of a nonconserved region of BCL2 confers a novel gain of function - suppression of apoptosis with concomitant cell proliferation. *Cancer Research* **56**, 2506-2509.

Van-Criekinge, W., Beyaert, R., Van de Craen, M., Van den Abeele, P., Schotte, P., De Valck, D., and Fiers, W. (1996) Functional characterization of the prodomain of interleukin-1-beta converting enzyme. *Journal Of Biological Chemistry* **271**, 27245-27248.

Vaux, D.L., Cory, S., and Adams, J.M. (1988) *Bcl2* gene promotes hematopoietic cell survival and cooperates with c-MYC to immortalize pre-b cells. *Nature* **335**, 440-442.

Vaux, D.L., Aguila, H.L., and Weissman, I.L. (1992) BCL2 prevents death of factor-deprived cells but fails to prevent apoptosis in targets of cell-mediated killing. *International Immunology* **4**, 821-824.

Veis, D.J., Sorenson, C.M., Shutter, J.R., and Korsmeyer, S.J. (1993) *Bcl2*-deficient mice demonstrate fulminant lymphoid apoptosis, polycystic kidneys, and hypopigmented hair. *Cell* **75**, 229-240.

Vidair, C.A., Chen, C.H., Ling, C.C., and Dewey, W.C. (1996) Apoptosis induced by X-irradiation of rec-myc cells is postmitotic and not predicted by the time after irradiation or behavior of sister cells. *Cancer Research* **56**, 4116-4118.

Vindelov, L.L., Christensen, I.J., and Nissen, N.I. (1983) A detergent-trypsin method for the preparation of nuclei for flow cytometric DNA analysis. *Cytometry* **3**, 323-327.

Voehringer, D.W., Story, M.D., O'Neil, R.G., and Meyn, R.E. (1997) Modulating Ca^{2+} in radiation-induced apoptosis suppresses DNA fragmentation but does not enhance clonogenic survival. *International Journal Of Radiation Biology* **71**, 237-243.

Waga, S., Hannon, G.J., Beach, D., and Stillman, B. (1994) The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. *Nature* **369**, 574-578.

Wagner, A.J., Kokontis, J.M., and Hay, N. (1994) Myc-mediated apoptosis requires wild-type p53 in a manner independent of cell cycle arrest and the ability of p53 to induce p21(WAF1/CIP1). *Genes & Development* **8**, 2817-2830.

Walker, N.I., Bennett, R.E., and Kerr, J.F.R. (1989) Cell death by apoptosis during involution of the lactating breast in mice and rats. *American Journal Of Anatomy* **185**, 19-32.

Walker, N.P.C., Talanian, R.V., Brady, K.D., Dang, L.C., Bump, N.J., Ferenz, C.R., Franklin, S., Ghayur, T., Hackett, M.C., Hammill, L.D., Herzog, L., Hugunin, M., Houy, W., Mankovich, J.A., McGuiness, L., Orlewicz, E., Paskind, M., Pratt, C.A., Reis, P., Summani, A., Terranova, M., Welch, J.P., Xiong, L., Moller, A., Tracey, D.E., Kamen, R., and Wong, W.W. (1994) Crystal structure of the cysteine protease interleukin-1-beta converting enzyme - a $(p20/p10)^2$ homodimer. *Cell* **78**, 343-352.

Wang, H.G., Rapp, U.R., and Reed, J.C. (1996b) BCL2 targets the protein kinase RAF1 to mitochondria. *Cell* **87**, 629-638.

Wang, K., Yin, X.M., Chao, D.T., Milliman, C.L., and Korsmeyer, S.J. (1996a) BID - a novel BH3 domain only death agonist. *Genes & Development* **10**, 2859-2869.

Wang, X.W., Forrester, K., Yeh, H., Feitelson, M.A., Gu, J.R., and Harris, C.C. (1994) Hepatitis B virus X protein inhibits p53 sequence-specific DNA binding, transcriptional activity, and association with transcription factor ERCC3. *Proceedings Of The National Academy Of Sciences Of The United States Of America* **91**, 2230-2234.

Wang, X.W., Yeh, H., Schaeffer, L., Roy, R., Moncollin, V., Egly, J.M., Wang, Z., Friedberg, E.C., Evans, M.K., Taffe, B.G., Bohr, V.A., Weeda, G., Hoeijmakers, J.H.J., Forrester, K., and Harris, C.C. (1995a) p53 modulation of TFIIH-associated nucleotide excision repair activity. *Nature Genetics* **10**, 188-195.

Wang, X.W., Vermeulen, W., Coursen, J.D., Gibson, M., Lupold, S.E., Forrester, K., Xu, G.W., Elmore, L., Yeh, H., Hoeijmakers, J.H.J., and Harris, C.C. (1996c) The XPB and XPD DNA helicases are components of the p53-mediated apoptosis pathway. *Genes & Development* **10**, 1219-1232.

Wang, Z.Q., Auer, B., Stingl, L., Berghammer, H., Haidacher, D., Schweiger, M., and Wagner, E.F. (1995b) Mice lacking ADPrt and poly(ADP-ribosyl)ation develop normally but are susceptible to skin-disease. *Genes & Development* **9**, 509-520.

White, K., Tahaoglu, E., and Steller, H. (1996) Cell killing by the drosophila gene *Reaper*. *Science* **271**, 805-807.

Williamson, D.J., Hooper, M.L., and Melton, D.W. (1992) Mouse models of hypoxanthine phosphoribosyltransferase deficiency. *Journal Of Inherited Metabolic Disease* **15**, 665-673.

Wilson, K.P., Black, J.A.F., Thomson, J.A., Kim, E.E., Griffith, J.P., Navia, M.A., Murcko, M.A., Chambers, S.P., Aldape, R.A., Raybuck, S.A., and Livingston, D.J. (1994) Structure and mechanism of interleukin-1-beta converting enzyme. *Nature* **370**, 270-275.

Wolf, D., Harris, N., Goldfinger, N., and Rotter, V. (1985) Isolation of a full-length mouse cDNA clone coding for an immunologically distinct p53 molecule. *Molecular And Cellular Biology* **5**, 127-132.

Wu, D.Y., Wallen, H.D., and Nunez, G. (1997) Interaction and regulation of subcellular localization of CED-4 by CED-9. *Science* **275**, 1126-1129.

Wu, L., Bayle, J.H., Elenbaas, B., Pavletich, N.P., and Levine, A.J. (1995) Alternatively spliced forms in the carboxy-terminal domain of the p53 protein regulate its ability to promote annealing of complementary single strands of nucleic acids. *Molecular And Cellular Biology* **15**, 497-504.

Wu, X.W., Bayle, J.H., Olson, D., and Levine, A.J. (1993) The p53 MDM2 autoregulatory feedback loop. *Genes & Development* **7**, 1126-1132.

Wu, Y., Liu, Y.G., Lee, L., Miner, Z., and Kuleszmartin, M. (1994) Wild-type alternatively spliced p53 - binding to DNA and interaction with the major p53 protein *in vitro* and in cells. *Embo Journal* **13**, 4823-4830.

Wyllie, A.H. (1980) Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature* **284** (No.5756), 555-556.

Wyllie, A.H., Kerr, J.F.R. and Currie, A.R. (1973) Cell death in the normal neonatal rat adrenal cortex. *Journal of Pathology* **111**, 255-261.

Xia, F., Wang, X., Wang, Y.H., Tsang, N.M., Yandell, D.W., Kelsey, K.T., and Liber, H.L. (1995) Altered p53 status correlates with differences in sensitivity to radiation-induced mutation and apoptosis in 2 closely related human lymphoblast lines. *Cancer Research* **55**, 12-15.

Xiao, H., Pearson, A., Coulombe, B., Truant, R., Zhang, S., Regier, J.L., Triezenberg, S.J., Reinberg, D., Flores, O., Ingles, C.J., and Greenblatt, J. (1994) Binding of basal transcription factor TFIIH to the acidic activation domains of VP16 and p53. *Molecular And Cellular Biology* **14**, 7013-7024.

Xiong, Y., Hannon, G.J., Zhang, H., Casso, D., Kobayashi, R., and Beach, D. (1993a) p21 is a universal inhibitor of cyclin kinases. *Nature* **366**, 701-704.

Xiong, Y., Zhang, H., and Beach, D. (1993b) Subunit rearrangement of the cyclin-dependent kinases is associated with cellular transformation. *Genes & Development* **7**, 1572-1583.

Xue, D., Shaham, S., and Horvitz, H.R. (1996) The *Caenorhabditis elegans* cell death protein CED-3 is a cysteine protease with substrate specificities similar to those of the human CPP32 protease. *Genes & Development* **10**, 1073-1083.

Yanagihara, K., Nii, M., Numoto, M., Kamiya, K., Tauchi, H., Sawada, S., and Seito, T. (1995) Radiation induced apoptotic cell death in human gastric epithelial tumor cells - correlation between mitotic death and apoptosis. *International Journal Of Radiation Biology* **67**, 677-685.

Yang, J., Liu, X.S., Bhalla, K., Kim, C.N., Ibrado, A.M., Cai, J.Y., Peng, T.I., Jones, D.P., and Wang, X.D. (1997) Prevention of apoptosis by BCL2: release of cytochrome c from mitochondria blocked. *Science* **275**, 1129-1132.

Yin, X.M., Oltvai, Z.N., and Korsmeyer, S.J. (1994) BH1 and BH2 domains of BCL2 are required for inhibition of apoptosis and heterodimerization with BAX. *Nature* **369**, 321-323.

Yin, Y.X., Tainsky, M.A., Bischoff, F.Z., Strong, L.C., and Wahl, G.M. (1992) Wild-type p53 restores cell cycle control and inhibits gene amplification in cells with mutant p53 alleles. *Cell* **70**, 937-948.

Yonish-Rouach, E., Resnitzky, D., Lotem, J., Sachs, L., Kimchi, A., and Oren, M. (1991) Wild-type p53 induces apoptosis of myeloid leukemic cells that is inhibited by interleukin 6. *Nature* **352**, 345-347.

Yonish-Rouach, E., Grunwald, D., Wilder, S., Kimchi, A., May, E., Lawrence, J.J., May, P., and Oren, M. (1993) p53-mediated cell death - relationship to cell cycle control. *Molecular And Cellular Biology* **13**, 1415-1423.

Young, L.S., Dawson, C.W. and Eliopoulos, A.G. (1997) Viruses and apoptosis. *British Medical Bulletin* **53** (No.3), 509-521.

Yuan, J.Y., Shaham, S., Ledoux, S., Ellis, H.M., and Horvitz, H.R. (1993) The *C. elegans* cell death gene *ced-3* encodes a protein similar to mammalian interleukin-1-beta converting enzyme. *Cell* **75**, 641-652.

Yuan, J.Y. and Horvitz, H.R. (1992) The *Caenorhabditis elegans* cell death gene *ced-4* encodes a novel protein and is expressed during the period of extensive programmed cell death. *Development* **116**, 309-320.

Zamzami, N., Marchetti, P., Castedo, M., Decaudin, D., Macho, A., Hirsch, T., Susin, S.A., Petit, P.X., Mignotte, B., and Kroemer, G. (1995) Sequential reduction of mitochondrial transmembrane potential and generation of reactive oxygen species in early programmed cell death. *Journal Of Experimental Medicine* **182**, 367-377.

Zauberman, A., Barak, Y., Ragimov, N., Levy, N., and Oren, M. (1993) Sequence-specific DNA binding by p53 - identification of target sites and lack of binding to p53MDM2 complexes. *Embo Journal* **12**, 2799-2808.

Zauberman, A., Lupo, A., and Oren, M. (1995) Identification of p53 target genes through immune selection of genomic DNA - the cyclin G gene contains 2 distinct p53 binding sites. *Oncogene* **10**, 2361-2366.

Zha, H.B., Aimesempe, C., Sato, T., and Reed, J.C. (1996b) Proapoptotic protein BAX heterodimerizes with BCL2 and homodimerizes with BAR via a novel domain (BN3) distinct from BH1 and BH2. *Journal Of Biological Chemistry* **271**, 7440-7444.

Zha, H.B., Fisk, H.A., Yaffe, M.P., Mahajan, N., Herman, B., and Reed, J.C. (1996a) Structure-function comparisons of the proapoptotic protein BAX in yeast and mammalian cells. *Molecular And Cellular Biology* **16**, 6494-6508.

Zha, J.P., Harada, H., Yang, E., Jockel, J., and Korsmeyer, S.J. (1996c) Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCLX(L). *Cell* **87**, 619-628.

Zhan, Q.M., Fan, S.J., Bae, I., Guillouf, C., Liebermann, D.A., O'Connor, P.M., and Fornace, A.J. (1994) Induction of BAX by genotoxic stress in human cells correlates with normal p53 status and apoptosis. *Oncogene* **9**, 3743-3751.

Zhang, W., McClain, C., Gau, J.P., Guo, X.Y.D., and Deisseroth, A.B. (1994) Hyperphosphorylation of p53 induced by okadaic acid attenuates its transcriptional activation function. *Cancer Research* **54**, 4448-4453.

Zhou, Q., Snipas, S., Orth, K., Muzio, M., Dixit, V.M., and Salvesen, G.S. (1997) Target protease specificity of the viral serpin CrmA - analysis of five caspases. *Journal Of Biological Chemistry* **272**, 7797-7800.

Zhu, H.J., Fearnhead, H.O., and Cohen, G.M. (1995) An ice-like protease is a common mediator of apoptosis induced by diverse stimuli in human monocytic THP.1 cells. *Febs Letters* **374**, 303-308.

Zhu, W.J., Cowie, A., Wasfy, G.W., Penn, L.Z., Leber, B., and Andrews, D.W. (1996) *Bcl2* mutants with restricted subcellular location reveal spatially distinct pathways for apoptosis in different cell types. *Embo Journal* **15**, 4130-4141.

Ziegler, A., Jonason, A.S., Leffell, D.J., Simon, J.A., Sharma, H.W., Kimmelman, J., Remington, L., Jacks, T., and Brash, D.E. (1994) Sunburn and p53 in the onset of skin cancer. *Nature* **372**, 773-776.

Zoratti, M. and Szabo, I. (1995) The mitochondrial permeability transition. *Biochimica Et Biophysica Acta-Reviews On Biomembranes* **1241**, 139-176.

Zou, H., Henzel, W.J., Liu, X.S., Lutschg, A., and Wang, X.D. (1997) APAF1, a human protein homologous to *C elegans* CED-4, participates in cytochrome c dependent activation of caspase-3. *Cell* **90**, 405-413.

Appendix I

Stock solutions.

Cell culture reagents.

Growth media (CM5-5):

Glasgow modification of Eagle's medium (Life Technologies) supplemented with the following reagents.

1. Non-essential amino acids (Life Technologies): Glycine (0.1mM), L-alanine (0.1mM), L-aspartic acid (0.1mM), l-asparagine (0.1mM), L-glutamic acid (0.1mM), L-proline (0.2mM) and L-serine (0.2mM).
2. L-glutamine (2mM, Life Technologies).
3. Sodium pyruvate (1mM, Life Technologies).
4. Foetal calf serum (5%, Sigma).
5. Newborn calf serum (5%, Globepharm).
6. β-mercaptoethanol (0.1mM, Sigma).
7. Recombinant leukaemia inhibitory factor (LIF)-supplemented medium (0.05 to 0.2%V/V, according to titration).

TVP (cell disaggregating solution):

0.025% (W/V) trypsin, 1mM EDTA and 1% chick serum (Life Technologies) in PBS. Sterilised by filtration (0.2μ, Gelman Sciences) and stored at -20°C.

1% Gelatin stock:

Gelatin (swine skin, Sigma) was dissolved in DDW, autoclaved, allowed to cool to room temperature, re-autoclaved and then stored at 4°C. This stock was diluted 1:10 with sterile DDW before use.

HAT selection medium:

CM5-5 medium was supplemented with 0.1mM hypoxanthine (Sigma), 20μM thymidine (Sigma) and 0.8 μM aminopterin (Sigma).

6 amino-6-mercaptopurine selection medium:

CM5-5 was supplemented with 10μg/ml 6 amino-6-mercaptopurine (6-TG, Sigma).

G418 selection medium:

CM5-5 was supplemented with 200μg/ml G418 (Life Technologies).

Vindelov reagents.

Solution A:

30μg/ml trypsin.

1mg/ml trisodium citrate.

0.5M Tris.

522μg/ml spermine tetrahydrochloride.

0.1% V/V Igepal CA-630 (Sigma).

pH 7.6.

Solution B:

0.5mg/ml trypsin inhibitor (type II-0, Sigma).

0.1mg/ml ribonuclease A (Sigma).

1mg/ml trisodium citrate.

0.5M Tris.

522μg/ml spermine tetrahydrochloride.

0.1% V/V Igepal CA-630 (Sigma).

pH 7.6.

Solution C:

0.4mg/ml propidium iodide.

1mg/ml spermine tetrahydrochloride.

1mg/ml trisodium citrate.

0.5M Tris.

522μg/ml spermine tetrahydrochloride.

0.1% V/V Igepal CA-630 (Sigma).

pH 7.6.

FISH reagents.

20xSSC:

3M NaCl.

0.3M Trisodium citrate.

70% formamide:

70% formamide.

2xSSC.

Blocking buffer:

4xSSC.

5% skimmed milk (Marvel).

Prepared freshly each day.

Avidin FITC:

4μ g/ml Avidin FITC DCS (Vector) freshly prepared in blocking buffer.

Biotinylated anti-avidin:

5μg/ml biotinylated anti-avidin (Vector) freshly prepared in blocking buffer.

Propidium iodide stock solution:

1mg/ml propidium iodide in DDW (dH_2O).

DAPI stock solution:

50μg/ml DAPI (Sigma) in dH_2O.

Vectormount:

1ml Vectormount antifadent (Vector).

1μl propidium iodide stock.

20μl DAPI stock.

p53 immunocytochemistry and immunohistochemistry reagents.

Lysis buffer:

150mM NaCl.

1% Nonidet P40 (Sigma).

0.5% sodium deoxycholate (Sigma).

0.1% SDS.

Monomer solution (Severn Biotech Ltd):

30% w/v acrylamide.

0.8% w/v bis acrylamide.

Running gel buffer (4x):

0.375M Tris.

pH 8.8.

Stacking gel buffer (4x):

0.125M Tris.

pH 6.8.

12.5% Running gel:

12.5 ml monomer solution.

7.5 ml running gel buffer.

0.3 ml 10% SDS.

9.6 ml DDW.

150 µl 10% ammonium persulphate.

10 µl TEMED.

4% Stacking gel:

0.88 ml monomer solution.

1.66 ml stacking gel buffer.

66 µl 10% SDS.

4.06 ml DDW.

33.4 µl 10% ammonium persulphate.

3.3 µl TEMED.

Loading buffer (2X):

0.125M Tris.

4% SDS.

20% v/v glycerol.

0.2M DTT.

0.02% bromophenol blue.

pH 6.8.

Tank buffer:

0.025M Tris.

0.192M glycine.

0.1% SDS.

pH 8.3.

Transfer buffer:

25mM Tris.

192mM glycine.

0.1% SDS.

20% methanol.

TBST:

0.01M Tris.

0.9% NaCl.

0.1% Tween 20.

pH 7.5.

TBS:

0.05M Tris.

0.9% NaCl.

pH 7.5.

Normal swine serum (NSS):

Normal swine serum (SAPU) was denatured for one hour at 65°C and then frozen at -20°C. An aliquot was defrosted and diluted 1:5 in TBS on the day of use.

DNA extraction reagents:

Cell lysis buffer:

0.05M Tris.

0.05M EDTA.

0.1M NaCl.

1% SDS.

0.005M DTT.

0.001M spermidine tetrahydrochloride.

Chloroform:

24 volumes chloroform.

1 volume iso-amyl alcohol.

TE:

10mM Tris.

1mM EDTA.

TNE:

10mM Tris.

1mM EDTA.

144mM NaCl.

Agarose gel electrophoresis reagents.

Tracking dye:

0.1% bromophenol blue.

0.1M EDTA.

50% V/V glycerol.

TBE buffer:

0.89M Tris.

0.89M boric acid.

0.002M EDTA.

pH 8.0

Agarose gels:

0.6-0.8% W/V agarose in TBE buffer.

Southern transfer reagents.

Alkali buffer:

1.5M NaCl.

0.5M NaOH.

Neutralising buffer:

1.5M NaCl.

0.5M Tris.

0.001M EDTA.

pH 7.2

20xSSC:

3M NaCl.

0.3M Trisodium citrate.

Hybridisation buffer:

0.1% SDS.

0.1% ficoll (Sigma).

0.1% polyvinyl-pyrrolidone (Sigma).

0.1% bovine serum albumin (Sigma).

10% dextran sulphate (Pharmacia).

50mM Tris.

10mM EDTA.

3xSSC.

pH 7.4.

Wash solution 1:

2xSSC.

0.1% SDS.

Wash solution 2:

0.5xSSC.

0.1% SDS.

Sequencing reagents.

Sequencing gel stock:

6% acrylamide/bis-acrylamide solution (Severn Biotech).

7M urea (Sigma).

1 x glycerol tolerant buffer (USB).

Stored at 4°C, protected from light.

Glycerol tolerant buffer (USB):

1.78M Tris.

0.58M taurine

0.01M EDTA.

Appendix II

Alphabetical list of common reagents and their suppliers.

Agarose; Sigma

Bench alcohol; Genta Medical

Boric acid; Sigma

Bromophenol blue; Fisher

Chloroform; Fisher

DEVD; Biomol

Dimethyl sulfoxide (DMSO); Sigma

DTT (dithiothreitol); Sigma

EDTA (Diaminoethanetetra-acetic acid), Sigma

Ethanol; Hayman

Ethidium bromide; Sigma

Formaldehyde; Fisher

Formamide: Fisher

Giemsa; Sigma

Gelatin; Sigma

Glycine; Sigma

Glycerol; Sigma

Glacial acetic acid; Fisher

HCl; Fisher

Hydrogen peroxidase; Sigma

Iso-amyl alcohol; Fisher

KCl; Fisher

Methanol; Fisher

NaCl; Fisher

NaOH; Fisher

Phenol; Sigma

Phosphate buffered saline (PBS); Life Technologies

Propan-2-ol; Fisher

Propidium iodide; Sigma

Proteinase K; ICN

Spermidine; Sigma

Spermine tetrahydrochloride; Sigma

Sodium dodecyl sulphate (SDS); ICN

Tris; Sigma

Trisodium citrate; Sigma

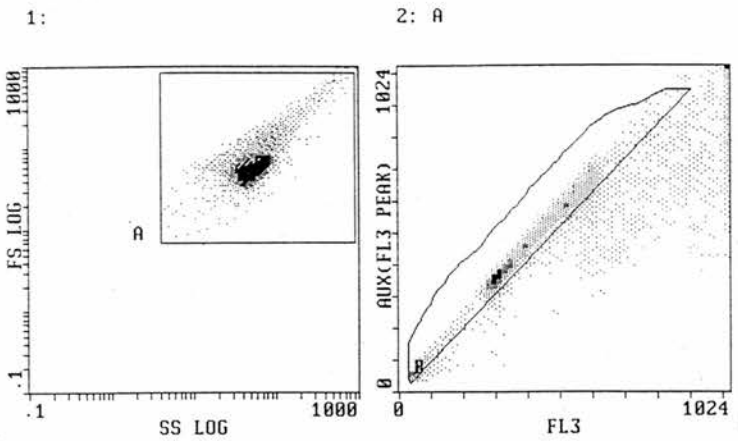
Trypsin; Sigma

Tween 20; Fisher

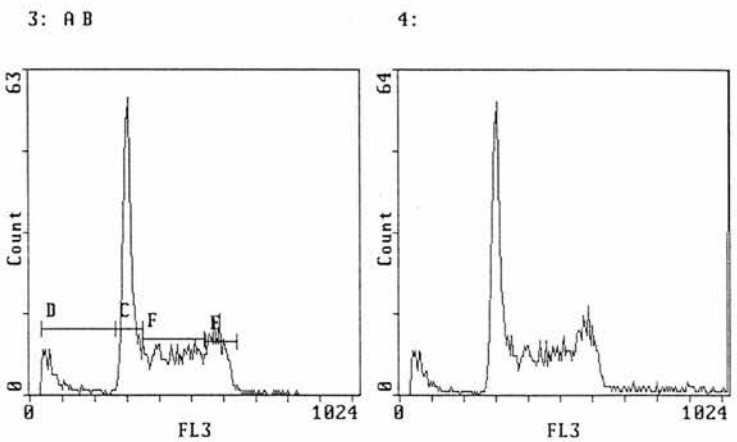
ZVAD; Bachem

Appendix III

Example of a typical flow cytometry analysis showing the gating protocols.



Hist	Region ID	%	Count	MnX	MnY
1	A A	98.9	12174	55.9	67.5
2	B B	78.6	9564	376.3	428.4



Hist	Region ID	%	Count	PkPosX	HPCV
3	D Hypodiploid	9.3	890	51.0	3.12
3	C G1	39.9	3812	307.0	3.24
3	E G2/M	17.1	1640	575.0	0.42
3	F S phase	32.2	3084	436.0	0.16